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Proteomic Analysis of Hepatic Tissue of Zebrafish (*Danio rerio*) Experimentally Exposed to Chronic Microcystin-LR

Minghua Wang,* Leo L. Chan,† Mengzi Si,* Huasheng Hong,* and Dazhi Wang*,[1]

*State Key Laboratory of Marine Environmental Science, Xiamen University, Xiamen 361005, People's Republic of China; and †School of Biological Sciences, The University of Hong Kong, Hong Kong Special Administrative Region, China

[1]To whom correspondence should be addressed. Fax: +86-0592-2186055. E-mail: dzwang@xmu.edu.cn.

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Microcystin-LR (MCLR) is the most toxic and most frequently encountered hepatotoxin in the aquatic environment. This study investigated the protein profiles of zebrafish (*Danio rerio*) livers chronically exposed to MCLR concentrations (2 or 20 μg/l) using the proteomic approach as well as cell ultrastructure, protein phosphatase (PP) activity, protein phosphatase 2A (PP2A) abundance, and toxin content analysis of the hepatic tissue. The results showed that, after 30-day exposure, the presence of MCLR strikingly enhanced toxin accumulation and the PP activity in zebrafish livers. However, the PP2A amounts were independent of toxin treatments. MCLR caused a noticeable damage to liver ultrastructure, a widespread swelling in the rough endoplasmatic reticulum and mitochondria was observed in the MCLR-exposed hepatocytes, and a honeycomb-like structure was formed in the treated nucleoli. Comparison of two-dimensional electrophoresis (2-DE) protein profiles of MCLR-exposed and nonexposed zebrafish livers revealed that the abundance of 22 proteins, measured by 2-DE, was remarkably altered in response to toxin exposure. These proteins were involved in cytoskeleton assembly, macromolecule metabolism, oxidative stress, and signal transduction, indicating that MCLR toxicity in fish liver is complex and diverse. Thus, proteomics provides a new insight into MCLR toxicity, that chronic toxicity of MCLR is different from acute toxicity, and we speculate that the reactive oxygen species pathway might be the main toxic pathway instead of the PP one. Moreover, even a low concentration of MCLR in water could significantly interrupt cellular processes, and more care should be taken in determining the criterion for MCLR content in drinking water.

Key Words: **microcystin-LR; protein phosphatase; proteomics; chronic toxicity; ultrastructure.**

Over the past few decades, the frequency and global distribution of toxic cyanobacterial blooms in eutrophic rivers, lakes, reservoirs, and recreational waters have significantly increased and become a worldwide concern. It is known that many species of cyanobacteria are able to produce cyanotoxins, which are harmful or toxic to aquatic organisms and animals including humans. Among cyanotoxins, microcystins (MCs), a group of cyclic nonribosomal polypeptides, are one of the most toxic groups due to their potent hepatotoxicity and the fact that they are probable tumor promoters (Chorus and Bartram, 1999). The group consists of more than 80 types with microcystin-LR (MCLR) as the most studied and the most toxic (Hoeger *et al.*, 2005). The toxic effects of MCLR on fish have been verified in a large number of studies involving histological, biochemical, and behavioral investigations (Baganz *et al.*, 2004; Fischer and Dietrich, 2000; Li *et al.*, 2004, 2007; Malbrouck *et al.*, 2003, 2004). MCs are taken up via an active transport mechanism similar to the bile acids, and they are also transported through the intestinal epithelium (Landsberg, 2002) and subsequently accumulated in liver (Malbrouck *et al.*, 2003). In humans, MCs have been implicated in contributing to the high incidence of primary liver cancers in certain areas of China where the primary sources of drinking water are ponds, lakes, and shallow wells (Ueno *et al.*, 1996). The typical toxicological action of MCs is to inhibit serine/threonine protein phosphatase (PP), followed by hyperphosphorylation of regulated proteins and a subsequent destruction of the hepatic cytoskeleton, leading to liver necrosis, apoptosis, and hemorrhage (Fischer and Dietrich, 2000; Gehringer, 2004). However, other toxicity mechanisms such as oxidative damage and disruption of osmoregulation are also reported (Blom and Jüttner, 2005; Chen *et al.*, 2006; Ding *et al.*, 2001; Mikhailov *et al.*, 2003). For instance, Ding *et al.* (2001) demonstrate that the production of reactive oxygen species (ROS) plays a crucial role in MC-induced disruption of cytoskeleton organization and consequent hepatotoxicity. Additionally, Gehringer (2004) postulates that MCLR might cause cellular events, e.g., cell death and proliferation, via the ROS and/or PP pathways. Overall, the precise mechanism of MCLR toxicity in organisms has not been well elucidated.

Global techniques such as proteomics provide effective strategies and tools for toxicological studies and are regarded as a powerful tool to investigate the cellular responses to toxicants (Dowling and Sheehan, 2006). In contrast to conventional biochemical methods, the proteomic approach offers great potential in identifying proteins involved in the

response of organisms to contaminants through massive comparison of protein expression profiles and helps to identify novel and unbiased biomarkers related to toxicity. With respect to MCs toxicity, proteomic-based approaches have proved to be valuable in identifying early responses to this toxin and, concomitantly, identifying the mechanisms of toxicity involved in the effects of MCLR on organisms including fish (Martins *et al*., 2009; Mezhoud *et al*., 2008a,b).

It should be noted that a large number of studies have been undertaken to investigate the acute toxicity of MCs (Fischer and Dietrich, 2000; Li *et al*., 2007; Malbrouck *et al*., 2003, 2004; Mezhoud *et al*., 2008a,b), but few efforts have been devoted to explore the chronic effects of MCs on organisms. Chronic exposure to MCs through drinking water is the main way for toxin accumulation in organisms including human beings, bearing in mind that the measured concentration of MCs is often a few micrograms per liter in the water of many large eutrophic lakes in the world (Chan *et al*., 2007; Gurbuz *et al*., 2009; Song *et al*., 2007). So, in this study, zebrafish (*Danio rerio*) were chronically exposed to MCLR concentrations (2 or 20 μg/l MCLR) for 30 days and the protein profiles of the liver cells of exposed and nonexposed zebrafish were analyzed using the proteomic approach, and the differentially expressed proteins were identified using matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometer (MS). The ultrastructural changes in liver cells as well as the toxin content, PP activity, and protein phosphatase 2A (PP2A) abundance in hepatic tissue were also investigated. The purpose of this study was to investigate the chronic toxicity of MCLR to zebrafish liver and identify new protein biomarkers so as to understand the damage mechanisms involved.

MATERIALS AND METHODS

Zebrafish Exposure Experiment

Zebrafish were acclimatized in aerated fresh water tanks for 20 days prior to the experiment at a water temperature of 25°C and a 12-h light/dark cycle and fed b.i.d., 0900 and 1500 h, with commercial dry bait. During the acclimation, fish were supplemented with artemia (about 40 mg per fish) three times a week to maintain nutritional status. Then, fish (weighing 0.7 ± 0.07 g) were randomly assigned to three experimental groups for exposure to two MCLR concentrations, 2 and 20 μg/l, and nonexposure. Each treatment included two groups with 40 fishes for each group. The experiments were carried out in glass tanks ($44 \times 30 \times 28$ cm^3) with 30 l filtered fresh water and lasted for 30 days under the same conditions as described above during acclimation. Each day, one-third of the aged water was renewed with fresh water containing 2 or 20 μg/l MCLR or none. No mortality was found in any treatment during the 30-day exposure. At the end of the experiment, 32 fishes were taken from each group and their livers were dissected and pooled together, and parts of them were immediately frozen in liquid nitrogen and stored at −80°C for proteomic, PP activity, PP2A abundance, and toxin content analysis. The remaining parts were fixed in 2.5% glutaraldehyde for cell ultrastructure analysis.

MCLR Analysis

The MCLR content of the hepatic tissues was analyzed according to the method (Moreno *et al*., 2005) with minor modification (Deblois *et al*., 2008). Briefly, freeze-dried tissue was homogenized in 0.6 ml of 85% methanol (MeOH) using an ultrasonic disrupter (Model 450; Branson Ultrasonics, Danbury, CT) at 4°C for 5 min. The homogenate was extracted at 4°C for 6 h and then centrifuged (8000 × g) at 4°C for 10 min, and the supernatant was recovered. The pellet was extracted again using the same procedure and the supernatant was pooled with that collected earlier and washed with the same volume of hexane for 4 h. The hexane layer was discarded and the MeOH layer was completely dried using Speed Vac. The dried liver extract was resolubilized in 100 μl of 50% MeOH for toxin analysis using high-performance liquid chromatography with a diode-array detector. The toxin separation was performed on a micropher C18 reverse phase column (3 μm) under isocratic conditions with a mobile phase of 10mM ammonium acetate and acetonitrile (7.4:2.6) for 20 min. The toxin content was quantified using an MCLR standard and expressed as micrograms cellular MCLR per milligram dry weight (DW).

PP Activity Analysis

Liver lysate was prepared using tissue protein extraction reagent (T-PER; Pierce Biotechnology Inc., Rockford, IL). Hepatic tissue (0.5 mg) was sonicated in 1 ml T-PER reagent and the supernatant was recovered by centrifugation (16,000 × g) at 4°C for 20 min. PP activity was analyzed according to the method of Fontal *et al*. (1999). Briefly, 35 μl of liver homogenate was mixed with 5 μl of $NiCl_2$ (40mM), 5 μl of 5 mg/ml bovine serum albumin (BSA; Sigma, St Louis, MO), and 35 μl of phosphatase assay buffer (50mM Tris-HCl, 0.1mM $CaCl_2$, pH 7.4) and incubated at 37°C for 10 min. Then, 120 μl of 100μM 6,8-difluoro-4-methylumbelliferyl phosphate (Sigma) was added and it was incubated at 37°C for another 30 min. PP activity was analyzed using a fluorescence microplate reader at 355 nm (excitation) and 460 nm (emission).

PP2A Abundance Analysis

The abundances of PP2A in MCLR-exposed and nonexposed zebrafish livers were performed using Western blot analysis. This assay was undertaken with the protein extracts, which were prepared as for the aforementioned PP activity analysis. Tissue homogenates containing 25 μg proteins were separated by 12.5% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions and transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore Co., Bedford, MA). Then, the membrane was blocked with 5% BSA for 2 h and incubated with an anti-PP2A rabbit monoclonal antibody (1:2000; Sigma) or an anti-β-actin mouse antibody (1:2000; Sigma) for 2 h at room temperature. Following three washes in PBS containing 0.05% Tween 20 (PBST), the membrane was incubated with a secondary anti-rabbit or mouse IgG (1:5000; Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at room temperature and then washed seven times in PBST. Finally, the membrane was incubated with peroxidase-labeled streptavidin-biotin complex (Nichirei Biosciences Inc., Tokyo, Japan) for 10 min. Images of the stained bands were captured using a Gel-documentation system on a GS-670 Imaging Densitometer from Bio-Rad (Hercules, CA). Images were saved in tagged image file format (TIFF) before analysis. Densitometric analysis for images was performed using the ImageMaster 2D Elite (GE Life Science, San Fransico, CA).

Ultrastructure Analysis

For ultrastructure analysis, fish livers were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.3) for 3 h at 4°C. The fixed livers were washed three times using 0.1M phosphate buffer (pH 7.3) at 20-min intervals with periodic agitation and then further fixed in 1% osmium tetroxide for 2 h at 4°C, followed by a phosphate buffer wash three times at 20-min intervals. After dehydration in alcohol, livers were embedded in Epon-Araldite. Ultra-thin sections (50–80 nm) were moved onto titanium grids, stained with uranyl acetate and lead citrate, and observed using a JEM 2100 Transmission Electron Microscope (JEOL Ltd., Tokyo, Japan). Image analysis was conducted using the ImageJ 1.36 program (NIH, Washington, DC).

Proteomic Analysis

Protein extraction. Frozen fish liver was homogenized in 0.6 ml of 20% TCA/acetone (wt/vol) lysis buffer with 20mM dithiothreitol (DTT) using

an ultrasonic disrupter. The supernatant was removed by centrifugation at 17,000 × g for 30 min at 4°C, and the pellet was washed twice with 80% acetone (vol/vol) and twice with ice-cold acetone with 20mM DTT. The pellet was recovered by centrifugation at 17,000 × g for 30 min at 4°C each time. Residual acetone was removed in a Speed Vac for about 5 min. The pellet was dissolved in 100 µl rehydration buffer containing 8M urea, 2% CHAPS, 2.8 mg/ml DTT, 0.5% immobilized pH gradient (IPG) buffer, and a trace of bromophenol blue. The solution was centrifuged at 20,000 × g for 30 min at 15°C and the supernatant was collected for two-dimensional electrophoresis (2-DE) analysis. The protein content was quantified using the 2-D Quant kit (GE Healthcare, San Fransico, CA).

2-DE analysis. Hundred micrograms of each protein sample was mixed with a rehydration buffer and then loaded onto IPG strips of linear pH gradient 4–7 (GE Healthcare). Rehydration and subsequent isoelectric focusing were conducted using the Ettan IPGphor III Isoelectric Focusing System (Amersham Biosciences, San Fransico, CA). Rehydration was performed overnight in a strip holder with 340 µl of rehydration buffer. After rehydration, isoelectric focusing was performed in the following manner: 2 h at 100 V, 2 h at 200 V, 1 h at 500 V, 2 h at 1000 V, 2 h at 4000 V, and 6 h at 8000 V. After the first dimension was run, each strip was equilibrated with about 10 ml equilibration buffer containing 50mM Tris (pH 8.8), 6M urea, 30% glycerol, 2% SDS, 1% DTT, and a trace amount of bromophenol blue for 17 min. The strip was then placed in fresh equilibration buffer containing 2.5% iodoacetamide (instead of DTT) for another 17 min. Subsequently, an 11.25% SDS-PAGE second dimension was performed. Electrophoresis was carried out at 10 mA/gel for 15 min, followed by a 6 h run at 200 V until the bromophenol blue front reached the edge of the gels. The gels were then visualized by silver staining.

Silver staining. Silver staining was performed following the method of Wang *et al.* (2009). Briefly, the gel was fixed for 2 h initially in a fixation solution containing 40% (vol/vol) ethanol and 10% (vol/vol) acetic acid. It was then sensitized in a solution containing 30% (vol/vol) ethanol, 0.2% (wt/vol) sodium thiosulphate, 6.8% (wt/vol) sodium acetate, and 0.125% (vol/vol) glutaraldehyde, followed by three Milli-Q water washes (5 min each time). Then, the gel was stained for 20 min in 0.25% (wt/vol) silver nitrate with 0.015% (vol/vol) formaldehyde and washed twice with Milli-Q water (0.5 min each time). The gel was developed in 2.5% (wt/vol) sodium carbonate containing 0.0074% (vol/vol) formaldehyde. The reaction was stopped with 1.5% (wt/vol) EDTA, disodium salt.

Image capture and analysis. Images were made using a Gel-documentation system on a GS-670 Imaging Densitometer from Bio-Rad and 2-DE electrophoretogram matching software. Images were saved in TIFF format before analysis. Computerized 2-DE gel analysis (spot detection, spot editing, pattern matching, and database construction) was performed using the ImageMaster 2D Elite (GE Life Science) and Melanie IV. There were three gels for samples from control or treated zebrafish livers. After spot detection and matching, spot intensities were normalized with total valid spot volume in order to minimize the nonexpression-related variations in spot intensity and hence accurately provide semiquantitative information across different gels. Spots normalization was done by analyzing the relative volume (volume percentage). A one-way ANOVA test was used to analyze spot intensities among different groups. Only protein spots showing a significance ($p < 0.05$) and at least a 2.0-fold difference in abundance (ratio of mean normalized spot volume of treated vs. control groups) were considered as upegulated or downregulated. These protein spots of interest were selected for identification by mass spectrometry.

Mass spectrometric analysis. Differentially expressed protein spots in MCLR-exposed and nonexposed livers were manually excised from 2-DE gels. The gel pieces were washed twice with 200mM ammonium bicarbonate in 50% acetonitrile/water (20 min at 30°C), then dehydrated using acetonitrile, and spun dry. In gel, trypsin digestion was performed by adding 20 ng/µl trypsin (Promega, Madison, WI) in 25mM ammonium bicarbonate overnight at 37°C. For MALDI-TOF/TOF MS analysis, 1 µl of the digest mixture was mixed on-target with 1 µl of 100mM α-cyano-4-hydroxy-cinnamic acid in 50% acetonitrile and 0.1% trifluoroacetic acid on the target plate before being dried and analyzed with a MALDI-TOF/TOF MS (4800 Proteomics Analyzer; Applied Biosystems, Foster City, CA). MALDI-TOF MS and TOF/TOF tandem MS were performed, and data were acquired in the positive MS reflector mode with a scan range from 900 to 4000 Da, and five monoisotopic precursors (S/N > 200) were selected for MS/MS analysis. For interpretation of the mass spectra, a combination of peptide mass fingerprints and peptide fragmentation patterns were used for protein identification in an NCBI nonredundant database using the Mascot search engine (www.matrixscience.com). All mass values were considered monoisotopic, and the mass tolerance was set at 75 ppm. One missed cleavage site was allowed for trypsin digestion, cysteine carbamidomethylation was assumed as a fixed modification, and methionine was assumed to be partially oxidized. Results with confidence interval % (CI %) values greater than 95% were considered to be a positive identification. The identified proteins were then matched to specific processes or functions by searching Gene Ontology (http://www.geneontology.org/).

Statistical Tests

All measurements were replicated at least three times, and the data were expressed as mean values ± SD. Statistical analysis was carried out using one-way ANOVA or an independent samples *t*-test to evaluate whether the means were significantly different among the groups. Significant differences were indicated at $p < 0.05$. Prior to one-way ANOVA, data were log transformed to meet ANOVA assumptions of normality and variance homoscedasticity.

RESULTS

Effect of MCLR on Toxin Accumulation, PP Activity, and PP2A Abundance in Zebrafish Liver

The toxin content, PP activity, and PP2A abundance of zebrafish livers at the end of this exposure experiment are shown in Figures 1 and 2. No MCLR was detected in MCLR nonexposed zebrafish livers, while this toxin treatment significantly enhanced toxin accumulation in the treated zebrafish livers (Fig. 1A). The toxin contents attained to 0.031 and 0.039 µg/mg DW, respectively, in the zebrafish livers exposed to 2 and 20 µg/l MCLR (Fig. 1A). However, the toxin content in the treated groups was independent of ambient MCLR concentrations ($p > 0.05$). Similarly, the hepatic PP activity was also enhanced by MCLR and the PP activity increased two times in zebrafish livers exposed to 20 µg/l MCLR (Fig. 1B). Next, using the antibody representing a specific interaction with PP2A, we performed Western blot analysis to examine the abundances of PP2A in the hepatocytes after 30 days of MCLR exposure. The relative ratio of each immunostained PP2A band to β-actin band was compared. Densitometric analysis indicated that the hepatic PP2A amount was independent of the ambient MCLR concentrations (Fig. 2A and 2B).

Effects of MCLR on Liver Ultrastructure in Zebrafish

The presence of MCLR resulted in a remarkable alteration in the liver ultrastructure of zebrafish (Fig. 3). Hepatocytes of nonexposed zebrafish usually contained few cisternae in the rough endoplasmatic reticulum (ER), which were presented in

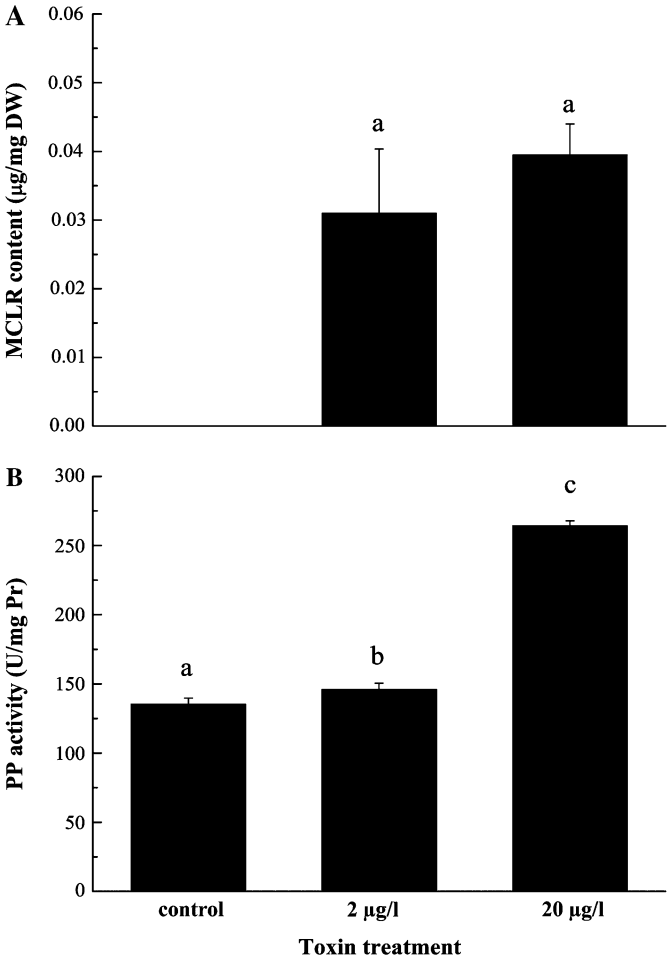

FIG. 1. MCLR contents (A) and PP activity (B) in the hepatic tissue of zebrafish *Danio rerio* after 30 days of MCLR exposure (control, 2, and 20 μg/l). Data are expressed as mean values ± SD ($n = 3$–4). Different letters indicate a statistically significant difference at $p < 0.05$.

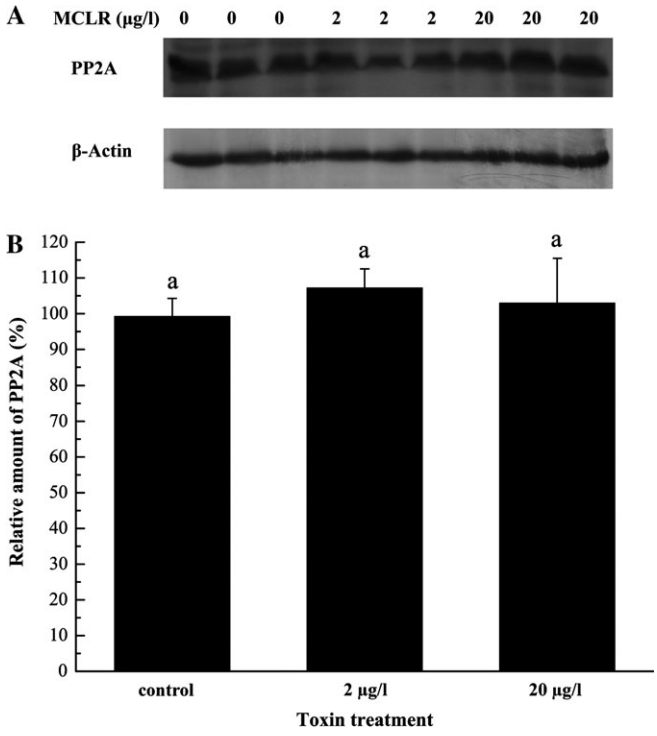

FIG. 2. PP2A amounts in the hepatic tissue of zebrafish *Danio rerio* after 30 days of MCLR exposure (control, 2, and 20 μg/l). Western blotting was performed with the specific antibody against PP2A and β-actin. (A) Western blotting images; (B) densitometric analysis of the intensities of the immunostained bands. Relative ratio of PP2A to β-actin was shown ($n = 3$). Different letters indicate a statistically significant difference at $p < 0.05$.

parallel layers or tightly associated with the mitochondria. Nucleoli were located in the center of the spherically shaped nuclei, the nucleoplasm exhibited very little heterochromatin, and the nucleolus displayed an electron density. Meanwhile, some of the cytoplasm was filled with glycogen (Fig. 3A). After 30-day exposure, a widespread swelling was observed in the rough ER, with the dilation or even vesiculation of cisternae, especially in the zebrafish livers exposed to 20 μg/l MCLR. The swelling of the mitochondrial matrix was also found in the exposed hepatocytes and many of them lost cristae and matrix in a progressive and dose-dependent manner. The most apparent change caused by MCLR was the formation of a honeycomb-like structure, which was electron dense with an intranucleolar structure forming a cordon or reticule.

Hepatic Protein Profiles of Zebrafish Exposed to MCLR

The 2-DE gels of MCLR-exposed and nonexposed zebrafish livers are shown in Figure 4, and quantitative spot comparisons were made with image analysis software. On average, more than 1000 protein spots were detected in each gel using silver staining and the ImageMaster 2D Elite software. Compared with the 2-DE gels of the nonexposed zebrafish livers, a total of 22 protein spots from the MCLR-exposed zebrafish livers was found to significantly alter in abundance ($\geq$ 2-fold or $\leq$ 0.5-fold; $p < 0.05$). Among these altered proteins, 3 protein spots disappeared in MCLR-exposed zebrafish livers (i.e., the 2 and 20 μg/l treatment groups), 7 protein spots were significantly upregulated, and 10 protein spots were noticeably downregulated in the 2 μg/l MCLR-exposed zebrafish livers (Table 1, Fig. 4). In the 20 μg/l MCLR-exposed group, 4 protein spots were found to remarkably upregulate and 11 protein spots were markedly downregulated (Table 1, Fig. 4). These altered protein spots were excised and submitted for identification using MALDI-TOF/TOF MS analysis. All the protein spots were successfully identified with CI % values greater than 95% (Table 1), and the matched proteins came from the NCBI database for zebrafish. The identified proteins were distinguished into 22 different proteins. Of them, nine proteins (spots 1, 6, 8, 10, 14, 15, 17, 19, and 22) were involved in metabolism, and five proteins (spots 4, 5, 7, 9, and 13) in proteolysis, and most of them presented serine-type endopeptidase activity. Two proteins were characterized as cell

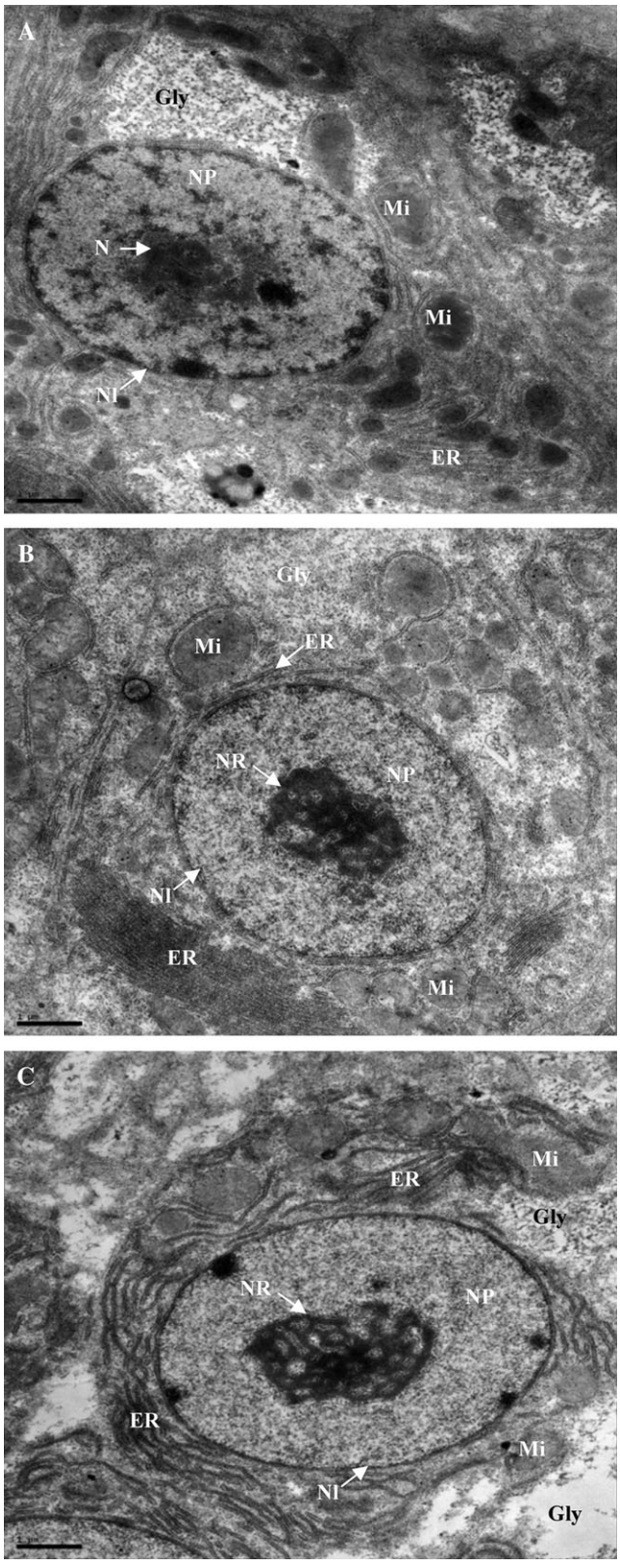

FIG. 3. Transmission electron micrographs of liver cells of the zebrafish *Danio rerio* after 30 days of MCLR exposure. (A) Control, (B) 2 μg/l MCLR, and (C) 20 μg/l MCLR. Marked features are: Nl, nucleus; N, nucleolus; NR, reticular nucleolus; NP, nucleoplasm; Mi, mitochondria; Gly, glycogen.

cytoskeleton proteins, corresponding to α-actinin 4 and profilin 2 like. The other six proteins were categorized into calcium/phospholipid binding, antioxidant defense, protein folding and other functional proteins.

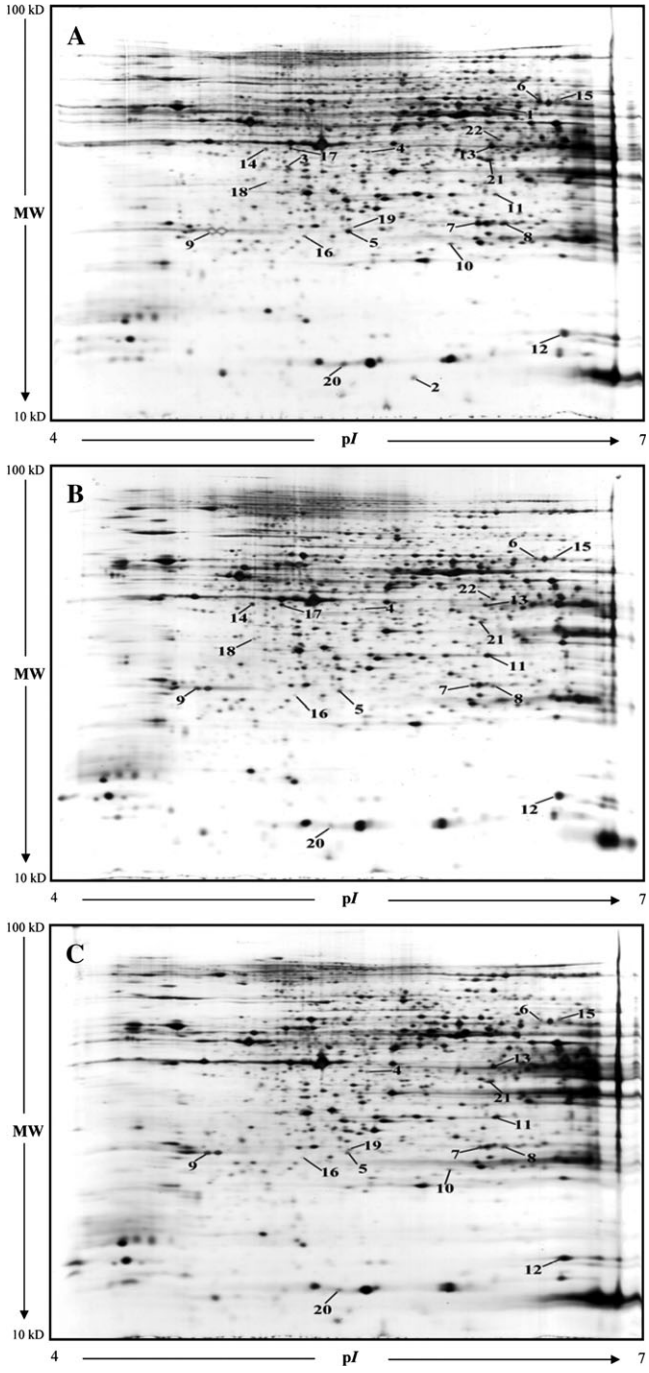

FIG. 4. Representative 2-DE gels of hepatic proteins in the zebrafish *Danio rerio* after the 30 days MCLR exposure. (A) Control, (B) 2 μg/l, and (C) 20 μg/l. Whole hepatic soluble proteins from zebrafish livers were separated by 2-DE and visualized by silver staining. The protein spots altered by MCLR exposure are labeled with numbers. The molecular weights (MW) and p*I* scales are indicated. Each gel is representative of three independent replicates.

TABLE 1
A Detailed List of Protein Spots Identified by MALDI-TOF/TOF MS from the Liver of the Zebrafish *Danio rerio* Following MCLR Exposure

Spot Id.	Protein identity	Accession number	MASCOT score (peptides)	Expectation value	MW/p*I*	Fold change 2μg/l	Fold change 20μg/l	Function category
Metabolism								
14	Zgc:136933	Q29R95	49 (1)	6.7e−5	41.55/4.95	2.28 ± 0.23	—	Methyltransferase activity
17	Suclg2 protein	Q6PHH4	109 (2)	2.7e−16	45.25/5.57	2.26 ± 0.34	—	ATP binding/ligase activity
8	Zgc:92030	Q6DG23	58 (1)	4.1e−5	39.10/6.77	0.40 ± 0.09	0.49 ± 0.03	Acyl-CoA binding/catalytic activity
15	Amylase, alpha 2A; pancreatic	Q6P5J0	109 (2)	8.4e−21	56.95/6.42	0.34 ± 0.09	0.34 ± 0.02	Cation binding/catalytic activity
10	Zgc:109929	Q504E5	32 (1)	1.6e−5	24.16/6.21	—	0.43 ± 0.06	Catalytic activity
19	6-Phosphogluconolactonase	Q68EK1	87 (2)	3.8e−13	11.78/5.84	—	3.74 ± 0.21	6-Phosphogluconolactonase activity
1	Pyruvate kinase	A8KC20	136 (3)	4.2e−22	58.97/5.86	D	D	Glycolysis
6	2-Hydroxyacyl-CoA lyase 1	Q6NYI5	55 (1)	8.4e−10	61.64/6.29	0.27 ± 0.09	0.21 ± 0.05	Fatty acid α-oxidation
22	Diphosphomevalonate decarboxylase	Q5U403	60 (1)	5.6e−12	44.60/5.97	2.10 ± 0.19	—	Cholesterol/lipid/steroid/sterol biosynthesis
Proteolysis								
5	Zgc:112160 protein	Q6DHD9	48 (1)	1.9e−5	28.16/5.54	0.33 ± 0.10	0.46 ± 0.04	Serine-type endopeptidase activity
9	Zgc:66382	Q7SX90	276 (2)	6.7e−30	26.38/4.94	0.45 ± 0.05	0.46 ± 0.03	Serine-type endopeptidase activity
7	Elastase 3 like	Q504D4	52 (1)	4.2e−10	21.43/5.91	0.33 ± 0.08	0.48 ± 0.09	Serine-type endopeptidase activity
13	Cpb1 protein	Q6NWD1	134 (2)	2.7e−30	46.41/5.83	0.29 ± 0.03	0.27 ± 0.01	Metallocarboxypeptidase activity
4	Zgc:91794	Q6GMH9	149 (2)	1.3e−19	46.97/5.48	0.39 ± 0.03	0.32 ± 0.03	Metallocarboxypeptidase activity
Cytoskeleton								
3	Actinin, alpha 4, partial	Q7SYE2	69 (1)	4.5e−12	21.86/5.05	D	D	Actin binding
20	Pfn2l protein (Profilin 2 like)	Q7ZVJ0	64 (1)	5.2e−10	15.08/5.63	0.13 ± 0.03	0.32 ± 0.04	Actin cytoskeleton organization
Other functions related								
11	Annexin 4	Q804G7	70 (2)	4.2 e−7	35.63/6.07	4.42 ± 0.60	4.49 ± 0.67	Calcium/phospholipid binding
12	Superoxide dismutase [Cu-Zn]	O73872	86 (2)	6.1e−11	15.95/6.11	2.27 ± 0.06	2.92 ± 0.03	Antioxidant defense
18	Peptidyl-prolyl cis-trans isomerase	Q499A7	62 (1)	3.4e−12	17.37/8.87	2.54 ± 0.45	—	Protein folding
2	Novel protein (Si:ch211-240l19.8)	Q5RI57	47 (1)	8.4e−10	15.02/5.23	D	D	Unknown
21	Zgc:111984	Q567K2	100 (2)	4.2e−11	31.17/5.98	0.33 ± 0.04	0.47 ± 0.02	Unknown
16	LOC100004902 protein	Q4V8Q9	192 (2)	7.9e−29	28.70/5.29	2.25 ± 0.06	2.76 ± 0.24	Unknown

Notes. MW, molecular weights.

D indicates a disappearance of this protein spot in the treatment group. The fold changes (mean values ± SD, $n = 3$) are indicated as compared to the controls. Only the fold changes ($\geq$ 2-fold or $\leq$ 0.5-fold) are shown. Values ≥ 2 indicate upregulations and ≤ 0.5 downregulations.

DISCUSSION

As potent hepatotoxins, the toxicity of MCs to aquatic organisms has been studied comprehensively (Li *et al*., 2004, 2007; Malbrouck *et al*., 2003, 2004; Mezhoud *et al*., 2008a,b). The toxic damage of MCs to hepatocyte cytoskeletons *in vivo* is reported. Li *et al*. (2004) note that the ultrastructure of common carp livers significantly changes after exposure to a subchronic dose of MCLR (50 μg/kg) for 28 days. The exposed hepatocytes present a swollen endomembrane system, dilation of cisternae of the rough ER and ER transformation into concentric membrane whorls, and numerous electron-lucent membrane-bound vacuoles. This is basically in line with our study that MCLR significantly enhanced toxin accumulation in the treated hepatocytes and consequently resulted in noticeable damage to liver ultrastructure. These results demonstrated that MCLR caused a significant disruption of cytoskeleton organization in the hepatocytes, which might interrupt hepatic metabolism. Moreover, our study showed that MCLR induced the formation of a honeycomb-like structure in the treated nucleoli. Similarly, Paris-Palacios and Biagianti-Risbourg (2006) report that copper contamination induces a particular nucleolar alteration by forming a network or honeycomb-like structure in roach and zebrafish livers, and they presume that the formation of this structure might be caused by Cu accumulation in the hepatocellular nucleoli. Thus, MCLR might have acted on the nucleoli of zebrafish hepatocytes, which subsequently interfered with DNA transcription. However, the exact mechanism resulting in the formation of honeycomb-like structure in the nucleoli is still unknown and deserves further investigation.

It is known that one of the first striking cytotoxicities caused by MCLR is cytoskeleton disruption, which is a primary response of the cells to toxicants, and this was also highlighted by cell ultrastructure study. The abundances of two cytoskeleton proteins, α-actinin 4 and profilin 2 like, significantly varied in exposed livers: α-Actinin 4 disappeared and profilin 2 like was downregulated. α-Actinin is a modular protein belonging to the spectrin superfamily that cross-links and bundles actin filaments in cells (Burridge and Feramisco, 1981). Kaplan *et al*. (2000) identify mutations in the actinin-4 gene causative for familial focal segmental glomerulosclerosis. Similarly, mice deficient in the actinin-4 gene show severe glomerular disease resembling focal segmental glomerulosclerosis (Kos *et al*., 2003). Profilins are 12- to 17-kDa ubiquitous eukaryotic proteins (Carlsson *et al*., 1976) and appear to affect actin dynamics (Schluter *et al*., 1997). Haarer *et al*. (1990) report that disruptions or deletions of the profilin gene in *Saccharomyces* result in slow growth, abnormal cell shape, multinucleated cells, and altered distribution of actin. Therefore, MCLR might have affected actin dynamics and microfilament network and subsequently disrupted cytoskeleton organization of hepatocytes.

Liver metabolism is known to be disturbed by MCs. In this study, nine proteins involved in metabolism were identified. MCLR caused an upregulation of 6-phosphogluconlactonase, an enzyme in the pentose phosphate pathway, and converts 6-phosphogluconolactone to 6-phosphogluconate (Collard *et al*., 1999). Amylase, alpha 2A is an enzyme that hydrolyzes 1,4-alpha-glucoside bonds in oligosaccharide and polysaccharides and thus catalyzes the first step in the digestion of dietary starch and glycogen (Kaczmarek and Rosenmund, 1977), and its abundance was significantly inhibited by MCLR. Particularly, pyruvate kinase, a key enzyme involved in glycolysis, was totally prohibited by MCLR. Hereby, MCLR caused a dysregulation of glycogen in the hepatocelluar ultrastructure and induced dysfunction of carbohydrate metabolism, which is in accordance with a previous study that MCLR attack results in depletion of the hepatocyte glycogen content, increasing demand for energy and an incidental cellular metabolic exhaustion (Mezhoud *et al*., 2008a). Also, MCLR influenced the activities of several enzymes related to lipid/fatty acid metabolism and transport, i.e., the abundances of 2-hydroxyacyl-CoA lyase 1 and zgc:92030 were strikingly suppressed by MCLR. However, the abundance of diphosphomevalonate decarboxylase was strongly enhanced. 2-Hydroxyacyl-CoA lyase 1 is a peroxisomal enzyme involved in fatty acid α-oxidation and thus could influence lipid metabolism. Fatty acid α-oxidation is the main degradation pathway for 3-methyl-branched fatty acids such as phytanic acid and the inhibition or deficiency of this enzyme causes an accumulation of phytanic acid, which is the only known abnormality in adult Refsum's disease (Wierzbicki *et al*., 2002). Zgc:92030 contains one acyl-CoA-binding domain and might be involved in lipid metabolism since acyl-CoA-binding proteins participate in acyl-CoA metabolism by binding long-chain acyl-CoAs in glycerolipid biosynthesis and in β-oxidation (Faergeman and Knudsen, 1997). Diphosphomevalonate decarboxylase performs the first committed step in isoprenes biosynthesis and subsequently participates in steroid and cholesterol biosynthesis (Hogenboom *et al*., 2004). We found that MCLR could affect the abundances of other functional proteins involved in organic acid metabolism. For instance, suclg2 protein, regulating the formation of succinate and ATP from succinyl-CoA and ADP in the tricarboxylic acid cycle (Allen and Ottaway, 1986), was prominently enhanced by MCLR. Additionally, MCLR significantly stimulated the abundance of zgc:136933, which is concerned with metabolic conversion. MCLR also remarkably suppressed production of zgc:109929, which is a homolog of the fumarylacetoacetate hydrolase (FAH) domain. FAH, the last enzyme of the tyrosine catabolic pathway, catalyzes the hydrolysis of fumarylacetoacetate into fumarate and acetoacetate. A deficiency in FAH will result in hereditary tyrosinemia type I, which is an autosomal recessive disorder, characterized by hepatic and renal dysfunction as well as by neurological crises (Dreumont *et al*., 2004). Overall, MCLR might cause hepatic dysfunction in zebrafish via

disturbing carbohydrate, lipid, and organic acid metabolism. A further challenge is to explore the exact mechanisms concerning MCLR toxicity in liver via combining metabonomics, considering that liver is one of the most important organs in material metabolism.

Our study demonstrated that MCLR influenced the proteolysis process in zebrafish livers since the abundances of zgc:112160 protein, zgc:66382, elastase 3 like, cpb1 protein, and zgc:91794 were strikingly decreased by toxin treatment. These proteins play key roles in the digestion of dietary proteins (Elliott and Elliott, 1997), and their inhibitions by MCLR are considered to be a sensitive effect of low-toxin concentrations in zebrafish and potentially lead to malabsorption of food substances and lower energy uptake. Lankoff and Kolataj (2001) also find that microcystin-YR significantly inhibits proteases in mouse hepatocytes. Claeyssens *et al.* (1995) report that MCLR inhibits protein synthesis in hepatocytes.

In this study, the abundance of superoxide dismutase [Cu-Zn] (Cu-Zn SOD), an important enzyme involved in antioxidant defense (McCord and Fridovich, 1988), was remarkably enhanced in MCLR-exposed cells, which was consistent with a previous study that ROS content and activities of several antioxidant enzymes including SOD are significantly induced in fish hepatocytes after 6-h exposure to 10 μg/l MCLR (Li *et al*., 2003). MCLR also increased the abundance of annexin 4 in exposed livers. Annexins belong to a large family of glycoproteins that bind both Ca^{2+} and negatively charge phospholipids (Gerke and Moss, 1997) and are considered as an important component of calcium signaling pathways. Studies show that certain animal annexins play an important role in cells during their response to oxidative stress (Rhee *et al*., 2000; Sacre and Moss, 2002; Tanaka *et al*., 2004). The annexin-like protein poxy5 from *Arabidopsis thaliana* is reported to play a protective role in fighting against stress response via peroxidase activity (Gidrol *et al*., 1996). Actually, this peroxidase activity exhibited by annexins is further exemplified by other studies (Gorecka *et al*., 2005; Jami *et al*., 2008). So, the upregulation of annexin 4 might be associated with MCLR-induced oxidative stress via peroxidase activity, and this protein might also act as a signaling function in the stress transduction pathway. Additionally, annexins and protein kinases C (PKCs) are two distinct families of ubiquitous cytoplasmic proteins involved in signal transduction and almost all annexins are both *in vitro* and *in vivo* substrates for PKCs (Dubois *et al*., 1996), which highlights that the upregulation of annexin 4 might correlate with activation of PKC activity due to oxidative stress. In fact, PKCs can be activated by oxidative stress and inhibited by antioxidants (Boscoboinik *et al*., 1991; Gopalakrishna *et al*., 1997). The effects of PKC phosphorylation and oxidation on signaling events have not yet been described under MCLR exposure. Nevertheless, it is possible that oxidation and phosphorylation represent alternative mechanisms for stimulating cellular responses relevant to MCLR chronically toxic effects.

Another interesting finding of this study was that no alteration of PP or PP2A abundances was observed in zebrafish livers, but the hepatic PP activity significantly increased. This suggests that PP activity might not be correlated with PP amount in the cells. Xing *et al.* (2008) reveal that protein phosphatase methylesterase-1 (a PP2A-specific methylesterase) directly binds to the active site of PP2A, which results in inactivation of PP2A by evicting the manganese ions required for the phosphatase activity of PP2A. In addition, the PP activity is also regulated by phosphorylation (Oliver and Shenolikar, 1998). Thus, variations of posttranslational modifications (e.g., methylation and phosphorylation) for PP appear to regulate formation of PP complexes and consequently affect its phosphatase activity in cells, which might be responsible for the mismatch between PP activity and PP abundance in livers. Nevertheless, the PP activity increased with an increasing MCLR concentration in our study, which was inconsistent with previous studies (Guzman *et al*., 2003; Malbrouck *et al*., 2003, 2004; Mezhoud *et al*., 2008a,b). Guzman *et al.* (2003) show that a lethal dose of MCLR profoundly inhibits PP activity in the nuclear compartment, but this inhibition is not detected in sublethal doses, which result in detectable changes in the phosphorylation of p53. Recently, Mezhoud *et al.* (2008b) investigate MCLR toxicity in medaka fish liver using proteomic approach and find rapid change of protein phosphorylation or levels of the cytosolic proteins after exposed to 1000 μg/l MCLR for 30–60 min. They postulate that variations of some proteins in phosphorylation are possibly in agreement with the inhibition of PP2A activity, although this inhibition is not observed (Mezhoud *et al*., 2008b). Thus, the toxic effect of MCLR on PP activity might be dose dependent and that the toxic effects of MCLR at a chronic dose might be different from those at an acute dose. Moreover, previous studies show that other targets for MCLR (e.g., the beta unit of ATP synthase and aldehyde dehydrogenase 2) do exist in organisms (Chen *et al*., 2006; Mikhailov *et al*., 2003), and this might interrupt the overall toxicity of MCLR via the inhibition of PP activity. Actually, a previous study has exemplified the mismatch between PP inhibition and MCs toxicity in crustacean, which indicates that other mechanisms, such as uptake, transport, detoxification, and the presence of other target sites, may modulate the overall toxicity for an animal and can offset or even reverse the specific PP inhibitory activity (Blom and Jüttner, 2005). Overall, the chronic effect of MCLR in this study was not consistent with the acute-dose effects noted in previous studies, and its chronic toxicity might be initiated in cells via the ROS pathway, instead of the PP pathway (Gehringer, 2004).

In conclusion, this study demonstrated that proteomics provides a potential tool for studying the biochemical mechanisms concerning MCLR toxicity in zebrafish livers. The proteomic analysis revealed that MCLR toxicity caused dysfunction of cytoskeleton assembly and macromolecule

metabolism and induced oxidative stress with a concomitant interference with cell signal transduction. The chronic toxicity of MCLR might initiate the ROS pathway, instead of the PP pathway, which is the main mechanism for MCLR acute toxicity. Annexin 4 might accompany or be involved in the ROS pathway, and it is possible that an integrated effects of oxidative stress and PKC phosphorylation on signal transduction might occur at the chronic MCLR level. However, the precise mechanisms (e.g., an exact function of annexins in MCLR toxicity and a possible correlation of ROS with PKC in signaling transduction) involved in MCLR chronic toxicity remain to be further investigated. Caution is necessary in utilizing PP inhibition assay to measure MCs content in fish tissues and evaluate MCs toxicity. Low concentrations ($\leq$ 2 μg/l) of MCLR in water could significantly interrupt cellular processes especially various metabolic processes in zebrafish livers, and more care should be taken in using the criterion "MCLR content in drinking water" since the guideline value for MCs in drinking water is only 1 μg/l (Falconer *et al*., 1999). We also noted that, in the table presenting differences in protein abundance (Table 1), data at the 2 μg/l level were almost identical to data at the 20 μg/l dose, and this might be attributable to the fact that toxin contents in the treated groups were independent of the ambient MCLR concentrations.

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REFERENCES

Allen, D. A., and Ottaway, J. H. (1986). Succinate thiokinase in pigeon breast muscle mitochondria. *FEBS Lett.* **194,** 171–175.

Baganz, D., Staaks, G., Pflugmacher, S., and Steinberg, C. E. W. (2004). A comparative study on microcystin-LR induced behavioural changes of two fish species (*Danio rerio* and *Leucaspius delineatus*). *Environ. Toxicol.* **19,** 564–570.

Blom, J. F., and Jüttner, F. (2005). High crustacean toxicity of microcystin congeners does not correlate with high protein phosphatase inhibitory activity. *Toxicon* **46,** 465–470.

Boscoboinik, D., Szewczyk, A., Hensey, C., and Azzi, A. (1991). Inhibition of cell proliferation by α-tocopherol. Role of protein kinase C. *J. Biol. Chem.* **266,** 6188–6194.

Burridge, K., and Feramisco, J. R. (1981). Non-muscle α-actinins are calcium-sensitive actin-binding proteins. *Nature* **294,** 565–567.

Carlsson, L., Nyström, L. E., Lindberg, U., Kannan, K. K., Cid-Dresdner, H., Lövgren, S., and Jörnvall, H. (1976). Crystallization of a non-muscle actin. *J. Mol. Biol.* **105,** 353–366.

Chan, W. S., Recknagel, F., Cao, H. Q., and Park, H. D. (2007). Elucidation and short-term forecasting of microcystin concentrations in Lake Suwa (Japan) by means of artificial neural networks and evolutionary algorithms. *Water Res.* **41,** 2247–2255.

Chen, T., Cui, J., Liang, Y., Xin, X. B., Young, D. O., Chen, C., and Shen, P. P. (2006). Identification of human liver mitochondrial aldehyde dehydrogenase as a potential target for microcystin-LR. *Toxicology* **220,** 71–80.

Chorus, I., and Bartram, J. (1999). In *Toxic Cyanobacteria in Water: A Guide to Public Health Consequences, Monitoring, and Management*. E & FN Spon on behalf of WHO, London.

Claeyssens, S., Francois, A., Chedeville, A., and Lavoinne, A. (1995). Microcystin-LR induced an inhibition of protein synthesis in isolated rat hepatocytes. *Biochem. J.* **306,** 693–696.

Collard, F., Collet, J., Gerin, I., Veiga-da-Cunha, M., and Van Schaftingen, E. (1999). Identification of the cDNA encoding human 6-phosphogluconolactonase, the enzyme catalyzing the second step of the pentose phosphate pathway. *FEBS Lett.* **459,** 223–226.

Deblois, C. P., Aranda-Rodriguez, R., Giani, A., and Bird, D. F. (2008). Microcystin accumulation in liver and muscle of tilapia in two large Brazilian hydroelectric reservoirs. *Toxicon* **51,** 435–448.

Ding, W. X., Shen, H. M., and Ong, C. N. (2001). Critical role of reactive oxygen species formation in microcystin-induced cytoskeleton disruption in primary cultured hepatocytes. *J. Toxicol. Environ. Health* **64,** 507–519.

Dowling, V. A., and Sheehan, D. (2006). Proteomics as a route to identification of toxicity targets in environmental toxicology. *Proteomics* **6**(20), 5597–5604.

Dreumont, N., Maresca, A., Khandjian, E. W., Baklouti, F., and Tanguay, R. M. (2004). Cytoplasmic nonsense-mediated mRNA decay for a nonsense (W262X) transcript of the gene responsible for hereditary tyrosinemia, fumarylacetoacetate hydrolase. *Biochem. Biophys. Res. Commun.* **324,** 186–192.

Dubois, T., Oudinet, J. P., Mira, J. P., and Russo-Marie, F. (1996). Annexins and protein kinases C. *Biochim. Biophys. Acta Mol. Cell. Res.* **1313,** 290–294.

Elliott, W. H., and Elliott, D. C. (1997). In *Digestion and Absorption of Food: Biochemistry and Molecular Biology*. Oxford University Press, New York.

Faergeman, N. J., and Knudsen, J. (1997). Role of long-chain fatty acyl-CoAs esters in the regulation of metabolism and cell signaling. *Biochem. J.* **323,** 1–12.

Falconer, I. R., Bartram, J., Chorus, I., Kuiper-Goodman, T., Utkilen, H., Burch, M., and Codd, G. A. (1999). Safe levels and safe practices. In *Toxic Cyanobacteria in Water* (I. Chorus and J. Bartram, Eds.), pp. 155–178. E & FN Spon, London, UK.

Fischer, W. J., and Dietrich, D. R. (2000). Pathological and biochemical characterization of microcystin-induced hepatopancreas and kidney damage in carp (*Cyprinus carpio*). *Toxicol. Appl. Pharmacol.* **164,** 73–81.

Fontal, O. I., Vieytes, M. R., Baptista de Sousa, J. M., Louzao, M. C., and Botana, L. M. (1999). A fluorescent microplate assay for microcystin-LR. *Anal. Biochem.* **269,** 289–296.

Gehringer, M. M. (2004). Microcystin-LR and okadaic acid-induced cellular effects: a dualistic response. *FEBS Lett.* **557,** 1–8.

Gerke, V., and Moss, S. E. (1997). Annexins and membrane dynamics. *Biochim. Biophys. Acta Mol. Cell. Res.* **1357,** 129–154.

Gidrol, X., Sabelli, P. A., Fern, Y. S., and Kush, A. K. (1996). Annexin-like protein from *Arabidopsis thaliana* rescues delta oxyR mutant of *Escherichia coli* from H_2O_2 stress. *Proc. Natl. Acad. Sci. U.S.A.* **93,** 11268–11273.

Gorecka, K. M., Konopka-Postupolska, D., Hennig, J., Buchet, R., and Pikula, S. (2005). Peroxidase activity of annexin 1 from *Arabidopsis thaliana*. *Biochem. Biophys. Res. Commun.* **336,** 868–875.

Gopalakrishna, R., Gundimeda, U., and Chen, Z. H. (1997). Cancer preventive selenocompounds induce a specific redox modification of cysteine-rich regions in calcium-dependent isoenzymes of protein kinase C. *Arch. Biochem. Biophys.* **348,** 25–36.

Gurbuz, F., Metcalf, J. S., Karahan, A. G., and Codd, G. A. (2009). Analysis of dissolved microcystins in surface water samples from Kovada Lake, Turkey. *Sci. Total Environ.* **407,** 4038–4046.

Guzman, R. E., Solter, P. F., and Runnegar, M. T. (2003). Inhibition of nuclear protein phosphatase activity in mouse hepatocytes by the cyanobacterial toxin microcystin-LR. *Toxicon* **41,** 773–781.

Haarer, B. K., Lillie, S. H., Adams, A. E. M., Magdolen, V., Bandlow, W., and Brown, S. S. (1990). Purification of profilin from *Saccharomyces cerevisiae* and analysis of profilin-deficient cells. *J. Cell Biol.* **110,** 105–114.

Hoeger, S. J., Hitzfeld, B. C., and Dietrich, D. R. (2005). Occurrence and elimination of cyanobacterial toxins in drinking water treatment plants. *Toxicol. Appl. Pharmacol.* **203,** 231–242.

Hogenboom, S., Tuyp, J. J. M., Espeel, M., Koster, J., Wanders, R. J. A., and Waterham, H. R. (2004). Human mevalonate pyrophosphate decarboxylase is localized in the cytosol. *Mol. Genet. Metab.* **81,** 216–224.

Jami, S. K., Clark, G. B., Turlapati, S. A., Handley, C., Roux, S. J., and Kirti, P. B. (2008). Ectopic expression of an annexin from *Brassica juncea* confers tolerance to abiotic and biotic stress treatments in transgenic tobacco. *Plant Physiol. Biochem.* **46,** 1019–1030.

Kaczmarek, M. J., and Rosenmund, H. (1977). The action of human pancreatic and salivary isoamylases on starch and glycogen. *Clin. Chim. Acta* **79,** 69–73.

Kaplan, J. M., Kim, S. H., North, K. N., Rennke, H., Correia, L. A., Tong, H. Q., Mathis, B. J., Rodriguez-Perez, J. C., Allen, P. G., Beggs, A. H., *et al.* (2000). Mutations in ACTN4, encoding alpha-actinin-4, cause familial focal segmental glomerulosclerosis. *Nat. Genet.* **24,** 251–256.

Kos, C. H., Le, T. C., Sinha, S., Henderson, J. M., Kim, S. H., Sugimoto, H., Kalluri, R., Gerszten, R. E., and Pollak, M. R. (2003). Mice deficient in alpha-actinin-4 have severe glomerular disease. *J. Clin. Invest.* **111,** 1683–1690.

Landsberg, J. H. (2002). The effects of harmful algal blooms on aquatic organisms. *Fish. Sci.* **10,** 113–390.

Lankoff, A., and Kolataj, A. (2001). Influence of microcystin-YR and nodularin on the activity of some proteolytic enzymes in mouse liver. *Toxicon* **39,** 419–423.

Li, L., Xie, P., Li, S. X., Qiu, T., and Guo, L. G. (2007). Sequential ultrastructural and biochemical changes induced *in vivo* by the hepatotoxic microcystins in liver of the phytoplanktivorous silver carp *Hypophthalmichthys molitrix*. *Comp. Biochem. Physiol. C: Pharmacol. Toxicol.* **146,** 357–367.

Li, X. Y., Chung, I. K., Kim, J. I., and Lee, J. A. (2004). Subchronic oral toxicity of microcystin in common carp (*Cyprinus carpio* L.) exposed to microcystis under laboratory conditions. *Toxicon* **44,** 821–827.

Li, X. Y., Liu, Y. D., Song, L. R., and Liu, J. T. (2003). Responses of antioxidant systems in the hepatocytes of common carp (*Cyprinus carpio* L.) to the toxicity of microcystin-LR. *Toxicon* **42,** 85–89.

Malbrouck, C., Trausch, G., Devos, P., and Kestemont, P. (2003). Hepatic accumulation and effects of microcystin-LR on juvenile goldfish *Carassius auratus* L. *Comp. Biochem. Physiol. C: Toxicol. Pharmacol.* **135,** 39–48.

Malbrouck, C., Trausch, G., Devos, P., and Kestemont, P. (2004). Effect of microcystin-LR on protein phosphatase activity and glycogen content in isolated hepatocytes of fed and fasted juvenile goldfish *Carassius auratus* L. *Toxicon* **44,** 927–932.

Martins, J. C., Leão, P. N., and Vasconcelos, V. (2009). Differential protein expression in *Corbicula fluminea* upon exposure to a *Microcystis aeruginosa* toxic strain. *Toxicon* **53,** 409–416.

McCord, J. M., and Fridovich, I. (1988). Superoxide dismutase: the first twenty years (1968–1988). *Free Radic. Biol. Med.* **5,** 363–369.

Mezhoud, K., Bauchet, A. L., Château-Joubert, S., Praseuth, D., Marie, A., François, J. C., Fontaine, J. J., Jaeg, J. P., Cravedi, J. P., Puiseux-Dao, S., *et al.* (2008a). Proteomic and phosphoproteomic analysis of cellular responses in medaka fish (Oryzias latipes) following oral gavage with microcystin-LR. *Toxicon* **51,** 1431–1439.

Mezhoud, K., Praseuth, D., Puiseux-Dao, S., François, J. C., Bernard, C., and Edery, M. (2008b). Global quantitative analysis of protein expression and phosphorylation status in the liver of the medaka fish (*Oryzias latipes*) exposed to microcystin-LR I. Balneation study. *Aquat. Toxicol.* **86,** 166–175.

Mikhailov, A., Härmälä-Braskén, A. S., Hellman, J., Meriluoto, J., and Eriksson, J. E. (2003). Identification of ATP-synthase as a novel intracellular target for microcystin-LR. *Chem. Biol. Interact.* **142,** 223–237.

Moreno, I. M., Molina, R., Jos, A., Picó, Y., and Cameán, A. M. (2005). Determination of microcystins in fish by solvent extraction and liquid chromatography. *J. Chromatogr. A* **1080,** 199–203.

Oliver, C. J., and Shenolikar, S. (1998). Physiologic importance of protein phosphatase inhibitors. *Front Biosci.* **3,** 961–972.

Paris-Palacios, S., and Biagianti-Risbourg, S. (2006). Hepatocytes nuclear structure and subcellular distribution of copper in zebrafish *Brachydanio rerio* and roach *Rutilus rutilus* (Teleostei, Cyprinidae) exposed to copper sulphate. *Aquat. Toxicol.* **77,** 306–313.

Rhee, H. J., Kim, G. Y., Huh, J. W., Kim, S. W., and Na, D. S. (2000). Annexin I is a stress protein induced by heat, oxidative stress and a sulfhydryl-reactive agent. *Eur. J. Biochem.* **267,** 3220–3225.

Sacre, S. M., and Moss, S. E. (2002). Intracellular localization of endothelial cell annexins is differentially regulated by oxidative stress. *Exp. Cell Res.* **274,** 254–263.

Schluter, K., Jockusch, B. M., and Rothkegel, M. (1997). Profilins are regulators of actin dynamics. *Biochim. Biophys. Acta Mol. Cell. Res.* **1359,** 97–109.

Song, L. R., Chen, W., Peng, L., Wan, N., Gan, N. Q., and Zhang, X. M. (2007). Distribution and bioaccumulation of microcystins in water columns: a systematic investigation into the environmental fate and the risks associated with microcystins in Meiliang Bay, Lake Taihu. *Water Res.* **41,** 2853–2864.

Tanaka, T., Akatsuka, S., Ozeki, M., Shirase, T., Hiai, H., and Toyokuni, S. (2004). Redox regulation of annexin 2 and its implications for oxidative stress-induced renal carcinogenesis and metastasis. *Oncogene* **23,** 3980–3989.

Ueno, Y., Nagata, S., Tsutsumi, T., Hasegawa, A., Watanabe, M. F., Park, H. D., Chen, G. C., and Yu, S. H. (1996). Detection of microcystins, a blue-green algal hepatotoxin in drinking water sampled in Haimen and Fusui, endemic areas of primary liver cancer in China, by highly sensitive immunoassay. *Carcinogenesis* **17,** 1317–1321.

Wang, D. Z., Lin, L., Chan, L. L., and Hong, H. S. (2009). Comparative studies of four protein preparation methods for proteomic study of the dinoflagellate *Alexandrium* sp. using two-dimensional electrophoresis. *Harmful Algae* **8,** 685–691.

Wierzbicki, A. S., Lloyd, M. D., Schofield, C. J., Feher, M. D., and Gibberd, F. B. (2002). Refsum's disease: a peroxisomal disorder affecting phytanic acid alpha-oxidation. *J. Neurochem.* **80,** 727–735.

Xing, Y. N., Li, Z., Chen, Y., Stock, J. B., Jeffrey, P. D., and Shi, Y. G. (2008). Structural mechanism of demethylation and inactivation of protein phosphatase 2A. *Cell* **133,** 154–163.