Microcystin-LR Induces Ceramide to Regulate PP2A and Destabilize Cytoskeleton in HEK293 Cells

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Received January 15, 2012; accepted April 10, 2012

TOXICOLOGICAL SCIENCES 128(1), 147–157 (2012)
doi:10.1093/toxsci/kfs141
Advance Access publication April 20, 2012

Microcystin-LR (MCLR) is one of the most common and most toxic members of the microcystins, which cause serious environmental disasters worldwide. Although the major toxicity of MCLR has been ascribed to its potent ability to inhibit protein phosphatase 1 and protein phosphatase 2A (PP2A), recent studies have suggested that MCLR may also perturb other important cellular processes, such as generation of ceramide. Ceramide is an essential second messenger in cells and regulates various cellular mechanisms, including PP2A activation and cytoskeleton destabilization. However, whether and how ceramide may mediate MCLR-induced cellular effects is unclear. We have previously reported that low concentrations of MCLR upregulate, rather than inhibit, PP2A activity in human embryonic kidney 293 (HEK293) cells. In this study, we provide evidence that MCLR induces ceramide generation in HEK293 cells and in mouse kidney. Furthermore, ceramide may mediate the MCLR-induced upregulation of PP2A activity and protein level of PP2A regulatory subunits in HEK293 cells. MCLR intoxication also causes the PP2A/B55α subunit to localize to the Golgi apparatus, and this process may also be mediated by ceramide. Importantly, ceramide may mediate cytoskeleton destabilization, cell detachment, and apoptosis induced by MCLR in HEK293 cells, whereas a ceramide synthase inhibitor, desipramine, protects cells from these changes. Our results suggest that ceramide may mediate MCLR-induced PP2A regulation and cytoskeleton destabilization.

Key Words: microcystin-LR; ceramide; PP2A; CAPP; cytoskeleton.

Toxic cyanobacterial blooms have become a serious global environmental concern. Microcystin-LR (MCLR) is one of the most toxic cyanobacterial toxins that pose severe threat to the health of humans and livestock. Target organs of MCLR include liver, kidney, brain, and intestines (Codd et al., 2005). However, compared with hepatotoxicity, other tissue-specific toxicities of MCLR are less well studied. To date, studies have established that MCLR is a potent inhibitor of protein phosphatase 1 and protein phosphatase 2A (PP2A), the two main cellular protein phosphatases (PPs) (MacKintosh et al., 1990). Moreover, MCLR affects the expression of multiple proteins and induces reactive oxygen species (ROS) in vivo and in vitro (Chen et al., 2005; Ding et al., 2000, 2001; Fu et al., 2005; Wang et al., 2010; Xing et al., 2008). Furthermore, MCLR induces cytoskeleton destabilization in cells and in animal models (Ding et al., 2000; Falconer and Yeung, 1992; Wang et al., 2010). However, the exact effect of MCLR on PPs, in the context of cellular response and autoregulation, is not clear. The exact mechanisms of MCLR-induced cytoskeleton destabilization also need to be fully studied.

PP2A is a major protein phosphatase in cells and is involved in many important cellular processes (Janssens and Goris, 2001). The holoenzyme of PP2A comprises a scaffold subunit (PP2A/A), a catalytic subunit (PP2A/C), and one of a variety of regulatory subunits (PP2A/B). PP2A/A and PP2A/C form the core enzyme of PP2A. PP2A/B determines the specific subcellular localizations, substrates, and functions of PP2A holoenzyme (Ahn et al., 2007; Cho and Xu, 2007). MCLR, on the other hand, directly binds to PP2A/C subunit and thus inhibits PP2A activity (Xing et al., 2006). However, our group and other groups have previously demonstrated that certain concentrations of MCLR stimulate, rather than inhibit, PP2A activity in vivo and in vitro (Li et al., 2011; Liang et al., 2011; Wang et al., 2010). These studies suggest that in addition to inhibiting PPs, MCLR may initiate other cellular mechanisms regulating PP2A activity.

Ceramide is an important lipid-like second messenger generated by cells and subsequently regulates many cellular processes, including PP2A activation, cytoskeleton destabilization, and apoptosis (Hannun and Obeid, 2008; Zeidan et al., 2008). The population of PP2A being activated by ceramide is termed ceramide-activated protein phosphatase (CAPP) (Dobrowsky et al., 1993). CAPP consists of PP2A AC and PP2A ABC-containing PP2A/B55α or PP2A/B56α (Galadari et al., 1998). To date, reports have shown that multiple extracellular stimuli, and ROS, induce ceramide generation (Hannun
and Obeid, 2008; Poli et al., 2004). Interestingly, it has also been reported that MCLR leads to the increase of sphingolipid levels in mouse serum, suggesting that MCLR could induce ceramide generation (Billam et al., 2008). Nevertheless, direct evidence of MCLR inducing ceramide generation is yet to be found.

A common consequence after MCLR treatment is cytoskeleton destabilization (Ding et al., 2000, 2001; Falconer and Yeung, 1992; Ohta et al., 1992; Wickstrom et al., 1995). Studies have found that MCLR causes hyperphosphorylation of proteins such as cytokeratin 8 and 18, two major intermediate filaments in hepatocytes (Ohta et al., 1992). Based on these findings, it is generally believed that because MCLR impairs the activity of PPs, the resulting hyperphosphorylation of cytoskeletal proteins should be a major cause of cytoskeleton destabilization, though experimental data about the cause-and-effect relationship are still unclear (Falconer and Yeung, 1992; Ohta et al., 1992; Wickstrom et al., 1995). Nevertheless, studies have also shown that ROS is critical for MCLR-induced cytoskeleton destabilization (Ding et al., 2000, 2001). Cotreated superoxide scavenger even prevents the MCLR-induced cytoskeleton destabilization (Ding et al., 2001). However, the exact mechanism underlying ROS-mediated cytoskeleton destabilization induced by MCLR is also not fully characterized. Thus, the exact mechanism of MCLR-induced cytoskeleton destabilization is still under debate. Whether inhibition of PPs is fully responsible for MCLR-induced cytoskeleton destabilization is an intriguing question. Actually, recent studies have revealed that ceramide pathway leads to cytoskeleton destabilization (Zeidan et al., 2008). Because MCLR might induce ceramide generation, we are interested to find whether ceramide participates in MCLR-induced cytoskeleton destabilization.

To study the role of ceramide in MCLR-induced toxicity, we hypothesize that MCLR may induce ceramide generation, and ceramide may thereby mediate PP2A regulation and cytoskeleton destabilization. To test this hypothesis, we cotreated HEK293 cells with MCLR and a ceramide synthase inhibitor, desipramine (DESI) (Alikhani et al., 2007). We chose HEK293 cells because the nephrotoxicity of MCLR is much less understood than its hepatotoxicity even though the kidney is an important and major target organ of MCLR. Also, we have previously shown the existence of MCLR in HEK293 cells after treatment, and certain concentrations of MCLR upregulate PP2A activity (Li et al., 2011). Of note, the MCLR concentrations commonly used in cell lines are much higher than those in pure chemical assays of MCLR and PP2A (Komatsu et al., 2007). Therefore, we chose MCLR concentrations according to previous studies from our group and other groups (Gan et al., 2010; Li et al., 2011). Moreover, we applied MCLR to mice at dosages according to those applied in other groups (Chen et al., 2005; Gaudin et al., 2008) to study the role of ceramide in MCLR-induced nephrotoxicity in vivo. In the present study, we show that MCLR induces ceramide generation, which may further mediate PP2A regulation and cytoskeleton destabilization. To our knowledge, this study may be the first to report MCLR-induced cellular effects mediated by ceramide and may contribute to a better understanding of the toxicity of MCLR.

**MATERIALS AND METHODS**

**Cell culture and treatments.** The HEK293 cell line was purchased from Cellbank of the Chinese Academy of Sciences (Catalog No. GNHu18). HEK293 cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO2. The cells were allowed to attach and grow for 24 h before the media were replaced with fresh media containing MCLR (Alexis), C6-ceramide (N-hexanoyl-o-sphingosine; Sigma-Aldrich), or DESI (Sigma-Aldrich), as indicated in the text. For DESI cotreatment, the cells were pretreated with DESI for 2 h before replacing the medium containing both DESI and MCLR. The control groups for each assay were replaced with fresh media during cell treatment. The beginning and ending of the treatments were within the logarithmic phase of cell growth. Prior to any cell lysate preparation, the cells were washed twice with ice-cold PBS, harvested, and gently rinsed with PBS four times via pelleting to fully remove culture media.

**Animals and tissue preparation.** All experiments were carried out in accordance with the ethics guidelines of the Zhejiang University Animal Experimentation Committee and were in complete compliance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Thirty-two healthy male ICR mice weighing 26 ± 1 g were purchased from the Experimental Animal Center, Zhejiang Academy of Medical Sciences. The mice were housed in an air-conditioned environment at 24 ± 1°C with a 12-h light/dark cycle and were allowed to acclimatize to their surroundings for 3 days before the experiments started.

The animals were randomly assigned to four groups. The mice in the control group were ip injected with saline on day 4. The mice in the treatment groups received MCLR at a single dose of 40, 60, and 80 µg/kg of body weight, respectively, on day 4 through ip injection. Six hours after MCLR treatment, the mice were killed, and their kidneys were dissected and fixed in formalin for 24 h. The kidneys were embedded in paraffin before being sectioned consecutively at 4 µm in thickness.

**PP2A activity assay.** PP2A activity was measured using an immunoprecipitation phosphatase activity assay. Briefly, 300 µg of total cell lysate from each group was prepared in a lysis buffer (50mM Tris–HCl, pH 7.5, 0.2% Triton X-100, 0.1mM EDTA, 0.5mM PMSF, 0.05% β-mercaptoethanol, 10% glycerol) with a fresh protease inhibitor complex (Roche) for 40 min. The PP2A/C was precipitated from cell lysates using 3 µg of PP2A/C antibody (clone 1D6, Upstate) with rotation for 12 h at 4°C, and then 40 µl of Protein A glycerol) with a fresh protease inhibitor complex (Roche) for 40 min. The PP2A/C was precipitated from cell lysates using 3 µg of PP2A/C antibody (clone 1D6, Upstate) with rotation for 12 h at 4°C, and then 40 µl of Protein A agarose beads was added for 2-h rotation at 4°C. Of note, this PP2A/C antibody does not block phosphatase activity. PP2A activity was measured by incubating the immunoprecipitated protein with the substrate RRA(pT)V A (Promega) for 5 min at 37°C while shaking at 1000 rpm. Next, 25 µl of the reacted solution was mixed with 100 µl of a molybdate dye/additive in a 96-well plate. The analysis of free phosphate yielded by each group was performed as previously described (Li et al., 2011). Finally, the phosphatase activity was normalized to the relative amount of immunoprecipitated PP2A/C, referring to the Western blotting quantification analysis by ImageJ. The data were from three independent assays.

**Western blotting.** Western blotting was used to test the protein levels. The cell extracts were prepared in a lysis buffer (50mM Tris–HCl, pH 7.5, 150mM NaCl, 15mM EDTA, 0.1% Triton X-100, 0.05% β-mercaptoethanol, 1mM PMSF) with a fresh protease inhibitor complex (Roche) on ice for 30 min. Vortexing occurred every 10 min. After the protein concentration
was examined using the Bradford method. 60 µg of the cell extract was loaded onto SDS-PAGE for electrophoresis and then transferred to nitrocellulose membranes. Primary antibodies applied at 4°C overnight were against the following: PP2A/A (Cell Signaling Technology), PP2A/C (Cell Signaling Technology), PP2A/B55α (Calbiochem), PP2A/B56α (BD Biosciences), α-tubulin (Abcam), vinculin (Abcam), Rac1 (Abcam), GAPDH (KANGCHEN), Bcl-2 (Bioworld), Bcl-xL (Bioworld), Bax (Bioworld). The concentration recommended by the manufacturers and also standard Western blotting procedures were applied. Figures representing three independent assays were shown.

**Immunofluorescence.** Immunofluorescence was used to visualize the subcellular localizations of the proteins of interest. When approximately 50–60% confluence was obtained on the coverslips, the cells were fixed with 4% formaldehyde for 10 min and blocked with 10% FBS/PBS (v/v) for 20 min. The cells were then incubated with antibodies against the following: Ceramide (Sigma-Aldrich), PP2A/B55α (Calbiochem), GM130 (BD Biosciences), vimentin (Cell Signaling Technology), α-tubulin (Abcam), Rac1 (Abcam), and vinculin (Abcam). The antibodies were diluted to the recommended concentration in 0.1% saponin/FBS/PBS (w/v) and incubated on coverslips at 25°C for 1 h. After washing the coverslips with 10% FBS/PBS, secondary antibodies of Alexa Fluor 488 goat anti-rabbit IgG conjugate or Alexa Fluor 633 goat anti-rabbit IgG conjugate and Alexa Fluor 546 goat anti-mouse IgG conjugate (Invitrogen) were applied at 25°C for 1 h. Nuclei and F-actin were stained with DAPI (Sigma) and phalloidin (Molecular Probes), respectively. Images were captured with a Zeiss confocal microscope (LSM 510 META) with uniform conditions among the control group and treatment groups, and figures representing ten randomly chosen fields were presented.

For apoptotic nuclei counting, more than 1000 DAPI-stained nuclei from each group were counted from random fields. Uniformly stained nuclei were scored as healthy cells. Condensed or fragmented nuclei were scored as apoptotic (Newhouse et al., 2004). To obtain unbiased counting, slices were counted, without knowledge of their prior treatment.

**Immunohistochemistry.** Antigen retrieval was performed in 0.01M sodium citrate solution. After 3% hydrogen peroxide treatment for 10 min, a primary antibody against ceramide (Sigma-Aldrich) was applied on the slices at 37°C for 1 h. HRP goat anti-mouse secondary antibody was used at 37°C for 40 min, before diaminobenzidine (DAB) solution was applied to visualize ceramide. Hematoxylin was used to stain the nuclei. Images were captured under a microscope, and representative images were shown.

**Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay.** Procedures were followed based on the instructions of the TUNEL kit used (TaKaRa, Japan). In brief, kidney slices were treated with 3% hydrogen peroxide and with protease K afterward. TUNEL solution was applied on the slices at 37°C for 1 h. HRP-conjugated anti-FITC solution and DAB solution were used, before the nuclei were stained with hematoxylin. Therefore, under microscope examination, the nuclei in healthy cells were a blue color, whereas the nuclei in apoptotic cells were a brown color. Representative images were taken and shown.

**Cell detachment assay.** The cell detachment assay was used to measure the detachment of cells after treatment (Widau et al., 2010). Equal amounts of cells were seeded onto 12-well plates. After treatment, one well of cells in each group was trypsinized and harvested to measure the total number of cells. The cells in other wells were washed three times in PBS at room temperature and shaken gently for 10 s. After washing, adherent cells were trypsinized and harvested to measure the remaining number of cells. All cell samples were counted using a Beckman Coulter cell counter. The proportion of cell detachment is defined as (the total number of each group—the remaining number of each group)/(the total number of each group). Data were from three independent experiments.

**Statistical analysis.** All data are shown as the mean ± SD from three independent experiments (n = 3). Pairwise comparisons between the means were tested using a Student’s t-test, two-tailed analysis; *p < 0.05; **p < 0.01; and ***p < 0.001.

## RESULTS

**MCLR-Induced Ceramide Generation**

To directly demonstrate that MCLR induces ceramide generation, HEK293 cells were treated with different concentrations of MCLR, with or without DESI, followed by an immunofluorescence assay using an anticeramide monoclonal antibody, which is a widely recognized method to detect ceramide generation (Abdel Shakor et al., 2004; Bieberich et al., 2003; Fernandes et al., 2011; Grassme et al., 2002). As shown in Figure 1A, ceramide fluorescence intensity was observed higher in 2 and 10µM MCLR treatment groups than in the control group, whereas 10µM DESI diminished the increase. To ensure the suitable concentration of DESI used...
in this study, different concentrations of DESI were used for cotreatment with 10µM MCLR. As shown in Figure 1B, HEK 293 cells in the 5µM DESI cotreatment group still retained slightly higher intensity of ceramide staining than those in non-MCLR and non-DESI treatment controls, although 10 and 20µM DESI cotreatment brought down the intensity of ceramide staining to basal level. These data suggest that MCLR may induce ceramide generation in HEK293 cells, and 10µM DESI is suitable for this study to inhibit this process. Immunohistochemistry assay on mouse kidney slices also showed elevated staining of ceramide in MCLR-treated mice (Fig. 1C), indicating that MCLR treatment could also induce ceramide generation in vivo.

Ceramide Was Involved in Cellular Regulation of PP2A Induced by MCLR

We have previously found that MCLR stimulates PP2A activity in HEK293 cells at relatively low concentrations, which prompted us to investigate whether the stimulation might be dependent on the ceramide-induced CAPP. Therefore, HEK293 cells cotreated with 10µM DESI and different concentrations of MCLR were tested for PP2A activity. As shown in Figure 2A, at a low concentration (2µM) of MCLR, the upregulation of PP2A activity (approximately 130%) was reversed to about 80% by DESI. At a higher concentration (10µM), about 20% of the remaining PP2A activity had been almost completely inhibited with the cotreatment of DESI.

Next, we tested whether MCLR affected the protein levels of the two regulatory subunits of CAPP, namely, PP2A/B55α and PP2A/B56α, that have been shown to be upregulated by ceramide. As shown in Figure 2B, the protein levels of PP2A/B55α and PP2A/B56α increased with the rise of the MCLR concentration. To investigate whether these changes were related to ceramide, we cotreated HEK293 cells with DESI and MCLR. As shown in Figure 2C, the upregulation of PP2A/B55α and B56α protein levels was diminished by DESI. Consistent with our previous study (Li et al., 2011), the protein levels of PP2A/A and PP2A/C were not affected by MCLR or by DESI. Noteworthy, a higher molecular band (approximately 1 kDa) higher of PP2A/C was observable in MCLR-treated groups. Because the molecular weight of MCLR is approximately 1kDa, this band is likely to be that of MCLR combined with PP2A/C, as discussed in our previous study (Li et al., 2011). Thus, these data suggest that ceramide may play an essential role in MCLR-induced cellular regulation of PP2A activity and the protein levels of PP2A/B55α and PP2A/B56α.

MCLR-Induced Changes of PP2A/B55α Subcellular Localization May Be Related to Ceramide

To further study the role of ceramide in cellular regulation of subcellular localization of PP2A induced by MCLR, we costained PP2A/B55α and GM130 for confocal immunofluorescence assay. As shown in Figure 3, PP2A/B55α, but not PP2A/B56α (supplementary data), was increasingly located immediately outside the nucleus with the increase in MCLR concentration. The morphology of the Golgi apparatus, as shown by GM130, was contracted under 10µM MCLR treatment. Furthermore, under the 10µM MCLR treatment, there was a colocalization of PP2A/B55α and GM130, suggesting that a portion of PP2A/B55α may locate at the Golgi apparatus. However, these changes were diminished by the cotreated DESI, as shown. To ensure that ceramide was capable of inducing these changes, cells treated with 25µM Cα-ceramide were tested. As shown in Figure 3, PP2A/B55α was more cytosolic.

FIG. 2. MCLR-induced PP2A activity and regulatory subunit protein level changes may be mediated by ceramide. (A) HEK293 cells cotreated with or without 10µM DESI together with different concentrations of MCLR for 24 h before PP2A activity was tested using the immunoprecipitation phosphatase assay. The relative PP2A activity compared with the control is shown. Data represent means ± SD (n = 3) *p < 0.05; **p < 0.01; ***p < 0.001 compared with the control group or between groups as indicated. (B) HEK293 cells were exposed under different concentrations of MCLR for 24 h. PP2A/B55α and PP2A/B56α protein levels from cell lysates were measured by Western blotting, where α-tubulin was used as the loading control. (C) HEK293 cells were cotreated with or without 10µM DESI, together with different concentrations of MCLR for 24 h. Protein levels of PP2A/A, PP2A/C, PP2A/B55α, and PP2A/B56α were detected by Western blotting. α-tubulin was used as the loading control.
and located more at the Golgi apparatus, which was also contracted by the C6-ceramide treatment. These results suggest that ceramide may be involved in the MCLR-induced PP2A/B55α localizing to the Golgi apparatus.

Ceramide May Mediate HEK293 Cell Cytoskeleton Destabilization Induced by MCLR

To address the role of ceramide in MCLR-induced cytoskeleton destabilization, we treated HEK293 cells with or without DESI together with MCLR for immunofluorescence study. Actin filaments, microtubules, and vimentin, which is a major type of intermediate filaments in HEK293 cells, were stained. As shown in Figure 4, MCLR at 10µM but not at 2µM induced morphological changes in HEK293 cells, as the cells showed a trend of contraction and rounding. Moreover, the 10µM MCLR treatment markedly decreased the polymerization of F-actin. Meanwhile, a contraction, but not depolymerization, of vimentin and microtubules was also observable. However, these effects were diminished in the presence of DESI. Next, we examined these cytoskeleton elements after the cells were treated with 25µM C6-ceramide for 8 h. As shown in Figure 4, the treatment of C6-ceramide mimicked the MCLR-induced changes of cytoskeleton and cell morphology. These data suggest that ceramide may be involved in the MCLR-induced destabilization of HEK293 cytoskeleton.

MCLR-Induced HEK293 Cell Detachment May Be Related to Ceramide

Both MCLR and ceramide have been shown to induce detachment of HEK293 cells (Li et al., 2011; Widau et al., 2011).
Here, we examined whether the cell detachment induced by MCLR was mediated by ceramide. HEK293 cells treated with different concentrations of MCLR, together with or without DESI, were tested to examine focal adhesion using a confocal microscopy assay. As shown in Figure 5A, the 10µM MCLR treatment decreased the localization of vinculin in the focal adhesion at the extensions of HEK293 cells. However, this change was diminished by the cotreatment of DESI. Consistent with the results above, DESI diminished the morphological changes induced by 10µM MCLR treatment. Of note, the protein level of vinculin in HEK293 cells was stable during treatment, as shown in Figure 5B. Next, we tested HEK293 cells treated with 25µM C₆-ceramide for 8 h. Similar to the 10µM MCLR treatment, vinculin was also decreased in the focal adhesion at cell extensions (Fig. 5C). Thus, these data suggest that MCLR-caused decrease of cell adhesion may be related to ceramide. In the cell detachment assay (Fig. 5D), cell detachment induced by 10µM MCLR treatment was also diminished by the cotreatment of DESI, which was consistent with the cell adhesion microscopy assay. Therefore, we infer from these results that the MCLR-induced cell detachment may be mediated by ceramide.

**Rac1 May Be Involved in MCLR-Induced Cellular Effect**

It has been established that cytosolic Rac1, a Rho family protein, is essential for actin polymerization and integrin
adhesion complex assembly (Etienne-Manneville and Hall, 2002). The nucleation of Rac1 is regulated by ceramide (Embade et al., 2000; Kim and Kim, 1998). In the effort to specify the role of ceramide in MCLR-induced cytoskeleton destabilization and cell detachment, we stained Rac1 protein after HEK293 cells were treated with different concentrations of MCLR. As shown in Figure 6A, Rac1 increasingly located to nuclei with the rise of MCLR treatment, which is consistent with the effect of ceramide on Rac1. Because the total Rac1 protein level remains stable (Fig. 6B), it could be referred that cytosolic Rac1 is decreased by MCLR treatment, which is consistent with the effect of MCLR on actin polymerization and cell adhesion. Thus, these data supported the rationale that ceramide may play a role in MCLR-induced cellular effects and might further suggest that Rac1 may be involved in these effects, including cytoskeleton destabilization and cell detachment.

Ceramide May Be Involved in MCLR-Induced Apoptosis

It has been documented that both MCLR and ceramide are capable of inducing apoptosis through Bcl-2 pathway (Chen et al., 2005; Fu et al., 2005; Hanson et al., 2008; Li et al., 2011; Van Hoof and Goris, 2003). To investigate whether ceramide is implicated in MCLR-induced apoptosis, we measured the protein levels of antiapoptotic protein Bcl-2 and proapoptotic proteins Bad and Bax in HEK293 cells treated with different concentrations of MCLR, with or without DESI. As shown in Figure 7A, MCLR treatment decreased the protein level of Bcl-2 and increased the protein level of Bad, whereas Bax protein level remained stable. However, DESI cotreatment diminished the protein level changes of both Bcl-2 and Bad, whereas Bax remained (Fig. 7B). MCLR treatment caused classic apoptotic fragmentation and condensation of nuclei in HEK293 cells (Fig. 7C). Quantification of apoptotic nuclei was performed with DAPI staining. As shown in Figure 7C, MCLR caused increasing rate of apoptotic nuclei, although DESI cotreatment protected cells from apoptosis. These data suggest that ceramide may be involved in MCLR-induced apoptosis in HEK293 cells. A TUNEL assay on mouse kidney slices was applied to detect MCLR-induced apoptosis in vivo. Consistent with our results of HEK293 cells, MCLR treatment caused an increase in apoptosis in mouse kidney (Fig. 7D).
DISCUSSION

To our knowledge, this study possibly provides the first direct evidence that MCLR induces ceramide generation in vitro and in vivo. Further, we show that MCLR induces multiple cellular effects through ceramide, including stimulation of PP2A activity, regulation of PP2A subunit protein levels and subcellular localization of PP2A/B55α, contraction of the Golgi apparatus, cytoskeleton destabilization, cell detachment, and apoptosis (Fig. 8).

It has been well established that ceramide stimulates PP2A (Janssens and Goris, 2001; Van Hoof and Goris, 2003). We have also previously shown that relatively low concentrations of MCLR stimulate PP2A activity (Li et al., 2011). In this report, we show that the stimulation of PP2A activity and the upregulation of two CAPP regulatory subunits by MCLR may be dependent on ceramide. These results suggest that in addition to the direct inhibition, MCLR may initiate regulation of PP2A through ceramide. Ceramide-induced upregulation of PP2A/B55α and PP2A/B56α has been shown to promote apoptosis through regulation of Bcl-2 and Bad in multiple types of cells, including HEK 293 cells (Hanson et al., 2008; Van Hoof and Goris, 2003). Our results are consistent with the function of CAPP, as MCLR-induced upregulation of PP2A/B55α and PP2A/B56α correlates with the proapoptotic change of Bad and Bcl-2 on protein levels and correlates with the increase of apoptosis. Therefore, we propose that MCLR-induced upregulation of PP2A/B55α and PP2A/B56α may be related to MCLR-induced apoptosis. Besides generating CAPP, MCLR is also found to be directly binding to PP2A/C in our study. One may concern the difference of binding affinity of PP2A/C to MCLR between low- and high-dose groups because the 1-kDa up-shift band is more intense in the 10µM MCLR group. Here, we propose that the different intensities of this band are more likely to be a result of different concentrations of MCLR being used and therefore presented in cells.

In CAPP models, PP2A/B56α has been shown to localize in mitochondria (Van Hoof and Goris, 2003). However, little is known about the effect of ceramide on subcellular localization of PP2A/B55α. Interestingly in this study, both MCLR treatment and C 6-ceramide treatment cause PP2A/B55α to localize to the Golgi apparatus in HEK293 cells, whereas DESI prevents this MCLR-induced change. Thus, our data suggest that the interaction between PP2A/B55α and the Golgi apparatus might be a novel MCLR-induced cellular effect. Moreover, this cellular effect may also be mediated by ceramide.

Cytoskeleton destabilization is a common consequence induced by MCLR on multiple types of cells (Ding et al., 2000; Li et al., 2011; Wickstrom et al., 1995). However, the exact mechanism of MCLR-induced cytoskeleton destabilization is under debate. Previous studies have suggested that MCLR-induced hyperphosphorylation of proteins, such as cytokeratin 8 and 18, leads to cytoskeleton destabilization (Ohta et al., 1992). However, other studies show that at certain stages of MCLR intoxication, ROS plays an essential role for MCLR-induced cytoskeleton destabilization, although the exact mechanism is also not fully defined (Ding et al., 2000, 2001). In this study, we show that 10µM MCLR decreases the polymerization of actin filaments and causes the contraction of microtubulin and vimentin. This phenotype of cytoskeleton destabilization is consistent with other studies (Ding et al., 2000; Zeidan et al., 2008). Importantly, cotreatment of DESI prevents these changes, suggesting that ceramide may participate in MCLR-induced cytoskeleton destabilization. Furthermore, a Rho family protein, Rac1, is nucleated at MCLR treatment. Because the ceramide-regulated nucleation of Rac1 would render its ability to progress actin polymerization (Embade et al., 2000; Etienne-Manneville and Hall, 2002; Kim and Kim, 1998), these results may reinforce the role of ceramide in MCLR-induced cytoskeleton destabilization. It is noteworthy that the PP2A activity is lower when HEK293 cells are cotreated with DESI and 10µM MCLR than when treated with 10µM MCLR only (Fig. 2). However, cytoskeleton is prevented at the cotreatment group (Fig. 4). It is presumable that eventually...
hyperphosphorylation of proteins would cause damage to the cytoskeleton, but at this particular stage, we propose that ceramide may be an important mediator of MCLR-induced cytoskeleton destabilization.

In addition to the in vitro study, we applied MCLR to mice in an in vivo model. We show that MCLR treatment induces higher level of ceramide and apoptosis in mouse kidney, which is consistent with our in vitro data and also in accordance with the results from others (Billam et al., 2008; Gaudin et al., 2008). Other groups have also shown that MCLR induces DNA damage, histopathological damages, and impairment of renal function in kidneys (Gaudin et al., 2008; Nobre et al., 2001). However, the nephrotoxicity of MCLR is still less understood than its hepatotoxicity and needs to be further studied.

Ceramide is a second messenger with various functions and is commonly induced by multiple cellular stresses. Although the generation of ceramide under MCLR treatment has been suggested by previous studies, the underlying mechanisms still remain elusive. Because it has been reported that MCLR induces oxidative stress, which is also a major inducer of ceramide (Poli et al., 2004; Zeidan and Hannun, 2010), we hypothesize that

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**FIG. 7.** MCLR-induced apoptosis may be mediated by ceramide. (A) HEK 293 cells were treated with different concentrations of MCLR for 24 h before lysis. Protein levels of Bcl-2, Bad, and Bax from the cell lysate were measured by Western blotting where α-tubulin was used as the loading control. (B) HEK 293 cells were cotreated with different concentrations of MCLR and 10µM DESI for 24 h. Protein levels of Bcl-2, Bad, and Bax were tested from the cell lysate, where α-tubulin was used as the loading control. (C) HEK 293 cells were treated with different concentrations of MCLR together with or without 10µM DESI for 24 h before the nuclei were stained with DAPI. Representative photomicrographs of nuclei morphology of HEK 293 cells treated with vehicle control (a), or 10µM MCLR (b), were shown. White arrows identified apoptotic nuclei. Apoptotic nuclei were counted. The percentage of apoptotic nuclei/total nuclei was presented in a graph. Data represent mean ± SD (n = 3) *p < 0.05; **p < 0.01 compared with the control or as indicated. (D) Mice were ip injected with saline as a vehicle control (a), MCLR at 60 µg/kg of body weight (b), or MCLR at 80 µg/kg of body weight (c), over a period of 6 h before the kidneys were dissected and fixed. Kidney slices were stained with TUNEL solution (brown) and hematoxylin (blue). Red arrows pointed to positive TUNEL staining. For full color figures, please see online manuscript.

**FIG. 8.** Schematic representation of ceramide-mediated cellular effects induced by MCLR.
oxidative stress rather than inhibition of PPs might be involved in the generation of ceramide. Thus, in addition to the known role of ceramide in PP inhibition, this study demonstrates the role of ceramide as an important mediator of cytotoxicity induced by MCLR.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

FUNDING

National Nature Science Foundation of China [No. 81172703] and Key Special Program on the ST of China for the Pollution Control and Treatment of Water Bodies [No. 2008ZX07421-001].

ACKNOWLEDGMENTS

We dearly thank Ms Yifan Zheng from School of Public Health, Zhejiang University and members in the laboratory of Professor Lihong Xu in support for an in vivo study. We also thank Chunmei Chang from Professor Wei Liu’s laboratory for discussion and advice on experimental design. We thank Guifeng Xiao, Wei Yin, and Shuangshuang Liu for expert technical assistance in immunofluorescence assays.

REFERENCES


phosphatases 1 and 2A from both mammals and higher plants. FEBS Lett. 264, 187–192.


