Microcystic Cyanobacteria Causes Mitochondrial Membrane Potential Alteration and Reactive Oxygen Species Formation in Primary Cultured Rat Hepatocytes

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Cyanobacteria contamination of water has become a growing public health problem worldwide. *Microcystis aeruginosa* is one of the most common toxic cyanobacteria. It is capable of producing microcystins, a group of cyclic heptapeptide compounds with potent hepatotoxicity and tumor promotion activity. The present study investigated the effect of microcystic cyanobacteria on primary cultured rat hepatocytes by examining mitochondrial membrane potential (MMP) changes and intracellular reactive oxygen species (ROS) formation in cells treated with lyophilized freshwater microcystic cyanobacteria extract (MCE). Rhodamine 123 (Rh-123) was used as a fluorescent probe for changes in mitochondrial fluorescence intensity. The mitochondrial Rh-123 fluorescence intensity in MCE-treated hepatocytes, examined using a laser confocal microscope, responded in a dose- and time-dependent manner. The results thus indicate that the alteration of MMP might be an important event in the hepatotoxicity caused by cyanobacteria. Moreover, the parallel increase of ROS formation detected using another fluorescent probe, 2′,7′-dichlorofluorescin diacetate also suggests the involvement of oxidative stress in the hepatotoxicity caused by cyanobacteria. The fact that MMP changes precede other cytotoxic parameters such as nuclear staining by propidium iodide and cell morphological changes suggests that mitochondrial damage is closely associated with MCE-induced cell injury in cultured rat hepatocytes. *Key words:* cyanobacteria, hepatocytes, hepatotoxicity, microcystin, mitochondrial membrane potential, reactive oxygen species. *Environ Health Perspect* 106:409–413 (1998). [Online 12 June 1998] *http://ehpnet1.niehs.nih.gov/docs/1998/106p409-413ding/abstract.html*

Contamination of water by toxic blooms of cyanobacteria (blue-green algae) has occurred widely in many regions of the world and poses a serious public health problem (*1,2*). Among the huge family of cyanobacteria, *Microcystis aeruginosa* is the most common toxic species. Microcystis is able to produce microcystins, a group of cyclic heptapeptide compounds with potent hepatotoxicity and tumor promotion activity (*3–5*).

At present, the exact mechanisms by which microcystins induce hepatotoxicity and tumor promotion have not been fully elucidated. One of the well-studied mechanisms is that microcystins are potent inhibitors of protein phosphatase 1 and 2A, leading to increased protein phosphorylation, which is directly related to their cytotoxic effects and tumor-promoting activity (*1,6*). There is also some preliminary evidence indicating that oxidative damage plays an important role in the hepatotoxicity of microcystins. An earlier study in our laboratory demonstrated that oxidative stress is implicated in the hepatotoxic effects of cyanobacteria extract in cultured rat hepatocytes (*7*), which is consistent with some other reports showing the inhibitory effects of antioxidants on the toxicity of microcystins (*8–10*).

Mitochondria are among the most important subcellular organelles in maintaining cellular structure and function by providing more than 80% of energy requirements through ATP production. Moreover, mitochondria are the main source of intracellular reactive oxygen species (ROS) formation and integrally involved in oxidative stress and cellular injury (*11,12*). Some earlier studies have shown that microcystins caused morphological and functional changes in mitochondria. For instance, Bhattacharya et al. (*13*) found that both toxic cyanobacteria and purified microcystins caused significant mitochondrial damage based on a tetrazolium dye colorimetric test in which tetrazolium salt was converted to formazan via mitochondria. At the ultrastructural level, microcystin-treated mitochondria underwent a series of changes, including dense staining, dilated cristae, and hydropic appearance devoid of electron-opaque deposits, which correlates with loss of coupled electron transport (significant inhibition of state 3 respiration) (*14–16*).

In recent years, the evaluation of mitochondrial membrane potential (MMP) changes has become a powerful tool for studying mitochondrial damage and its role in cellular injury. Rhodamine 123 (Rh-123) is one of the most frequently used fluorescent probes. Mitochondrial fluorescent intensity correlates quantitatively with MMP changes (*17–19*). The present study was designed to study the effect of microcystic cyanobacteria extract (MCE) on primary cultured rat hepatocytes by examining the changes in MMP and intracellular ROS formation. Our results suggest that mitochondrial depolarization is closely associated with cellular injury induced by cyanobacteria.

Materials and Methods

Chemicals. William's medium E, rhodamine 123 (Rh-123), collagenase, Hepes buffer, penicillin, and streptomycin were all purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was from Cytosystems (Castle Hill, NSW, Australia). Propidium iodide (PI) and 2′,7′-dichlorofluorescin diacetate (DCFH-DA) were from Molecular Probes (Eugene, OR).

Collection of cyanobacteria sample. The cyanobacteria were collected in early autumn 1996 during a water bloom period from the Dianshan Lake, which is one of the main water sources for Shanghai, the biggest city in the People's Republic of China. Microscopic examination revealed that the water blooms were dominated by *Microcystis aeruginosa* (>90%).

Extraction of toxins. We extracted toxins according to the method described by Harada et al. (*20*) with modifications. As more than 90% of algae were *Microcystis aeruginosa*, it is believed that microcystins were the main toxins extracted. Briefly, lyophilized algae cells (25 mg) were first dissolved in 2.5 ml *n*-butanol:methanol:water (1:4:15, v/v/v) with high-speed stirring at room temperature for 1 hr, followed by centrifugation at 16,000*g* for 30 min. The precipitant was reextracted two more times as described above. The supernatant from three extractions was pooled and evaporated to dryness at 56°C, and then dissolved in 5 ml 20% methanol. The extracted

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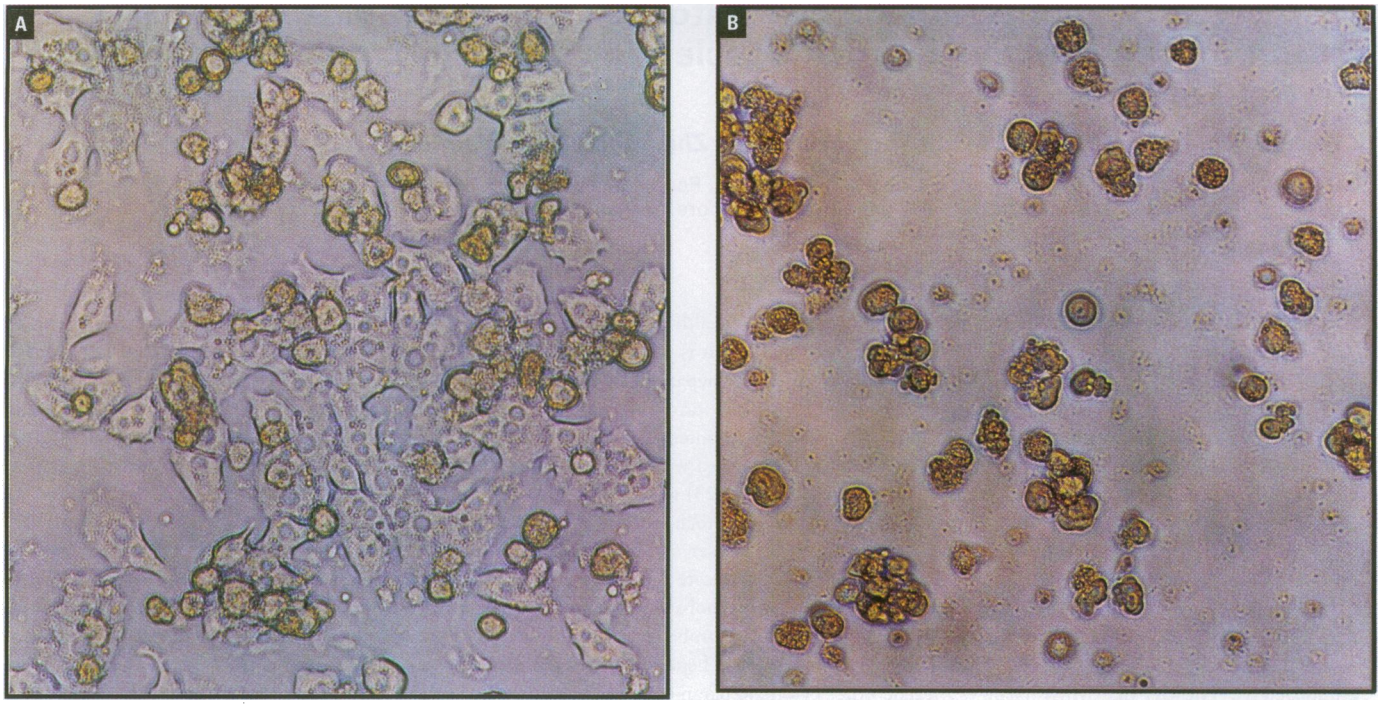

Figure 1. Morphological changes of cultured rat hepatocytes with a 12-hr incubation: (A) control hepatocytes and (B) cells treated with a high concentration of MCE (equivalent to 125 µg lyophilized algae cells/ml). Cells were examined using an inverted light microscope, ×100.

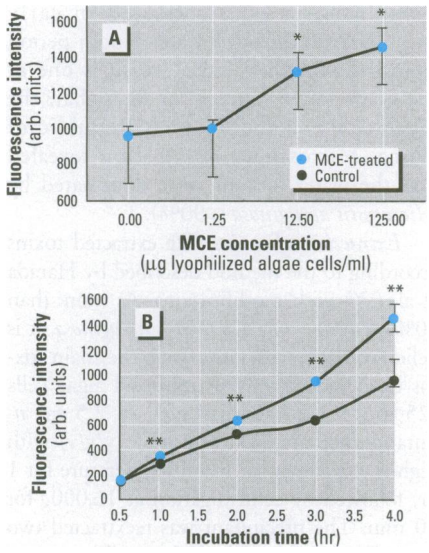

Figure 2. Microcystic cyanobacteria extract (MCE)-induced elevation of DCF fluorescence in cultured rat hepatocytes: (A) dose response and (B) time-course. The reaction took place with 1×10^5 hepatocytes and 1 µM DCFH-DA in 3 ml phosphate-buffered saline, and data are means ± standard deviations (n = 3). In the dose–response study, hepatocytes were exposed to three different concentrations of MCE for 4 hr; *p<0.05 compared to the control group (one-way ANOVA with Scheffe's test). In the time-course study, the hepatocytes were incubated with or without a high concentration of MCE (equivalent to 125 µg lyophilized algae cells/ml) up to 4 hr; **p<0.001 compared to the control group at each time point (Student's t-test).

fraction was then passed through a preconditioned Sep-Pak C18 cartridge (3-ml tube, Supelco, Bellefronte, PA) by washing with 3 ml 20% methanol and was then eluted with 10 ml methanol. Finally, the methanol elute was evaporated to dryness at 56°C and dissolved in 2 ml of distilled, deionized water. The final MCE was kept at 4°C for subsequent tests.

Liver perfusion and primary rat hepatocyte culture. Liver perfusion and primary rat hepatocyte culture were carried out as described by Shen et al. (*21*). The cells were plated at a density of 4×10^6 cells/75-cm^2 flask (Corning, NY). After preincubation for 2 hr in 10 ml Williams' Medium E supplemented with 10% FBS, the flasks were washed with prewarmed Hepes buffer (pH 7.4) to remove the unattached dead cells. The hepatocytes were then incubated in serum-free Williams' Medium E with various treatments.

Morphological changes of treated hepatocytes. The morphological changes of cultured rat hepatocytes were examined under an inverted microscope (Diaphot-TMD; Nikon, Tokyo, Japan) and directly photographed without any staining after 12 hr of incubation.

Detection of intracellular ROS formation. MCE-induced ROS formation was measured by using a fluorescent probe, DCFH-DA, as established earlier in our laboratory (*22*). The basic reaction mixture contained 1×10^5 hepatocytes and 1 µM DCFH-DA in 3 ml phosphate-buffered saline. In the dose–response study, cells were treated with low, moderate, and high concentrations of MCE (equivalent to 1.25, 12.5, and 125 mg lyophilized algae cells/ml, respectively) for 4 hr. In the time-course study, hepatocytes were coincubated with MCE equivalent to 125 µg lyophilized algae cells/ml at 37°C up to 4 hr. The fluorescence intensity was monitored using a Perkin-Elmer spectrofluorometer LS-5B with excitation wavelength at 485 nm and emission wavelength at 530 nm.

Determination of MMP with Rh-123. Rh-123 is taken up into cells and localized into mitochondria because of the high negative electrical potential across the mitochondrial membrane. The diffusion and accumulation of Rh-123 in mitochondria is proportional to the degree of MMP (*17–19*). On the other hand, PI is unable to penetrate the plasma membrane of viable cells but labels the nuclei of nonviable cells (*23*). Hepatocytes were first cultured in coverglass chambers (Nunc, Naperville, IL) for 24 hr before being treated with MCE. For the dose–response study, hepatocytes were treated with low, moderate, and high MCE (equivalent to 1.25, 12.5, and 125 µg lyophilized algae cells/ml, respectively) for 60 min. For the time-course study, cells were exposed to a high concentration of MCE (equivalent to 125 µg lyophilized algae cells/ml) for up to 120 min. Rh-123 (final concentration 6 µg/ml) and PI (final

concentration 1.5 μM) were added simultaneously, 30 min before the end of treatment. The hepatocytes were washed with phosphate-buffered saline twice to remove the MCE and the fluorescence probes and immediately evaluated under a laser scanning inverted confocal microscope (Carl Zeiss LSM 410, Germany). Rh-123 was excited at 488 nm laser line and the emission signal was observed with a combination of a 510 nm dichronic mirror and a 515-516 nm cut-off filter. PI was excited at 514 nm and the emission signal was observed with a LP-590 nm filter. A heated platform was fitted to the microscope and set at 37°C through the analysis.

Statistical analysis. Data are presented as means ± SD from at least three sets of measurements and were analyzed using one-way ANOVA with Scheffe's test or Student's *t*-test. A *p*-value <0.05 was considered statistically significant.

Results

MCE-induced Morphological Changes in Primary Cultured Rat Hepatocytes

The morphological changes in cells treated with MCE became visible from 3 hr onward. The typical alterations under light microscopy after 12 hr of treatment are shown in Figure 1. The majority of the untreated cells after 12 hr of incubation remained as a monolayer with polygonal or oval shapes, extended pseudopodia, and visible nuclei (Fig. 1A). In contrast, cells treated with MCE for 12 hr became spherical and detached from the cultured flasks, without any pseudopodia (Fig. 1B).

MCE-enhanced ROS Formation in Primary Cultured Rat Hepatocytes

Both the dose- and time-dependent changes of ROS production induced by MCE in primary cultured rat hepatocytes were studied, and the results are shown in Figure 2. In the dose–response study, a significant difference from the control group was noted in cells treated with moderate and high concentration of MCE (equivalent to 12.5 and 125 μg lyophilized algae cells/ml, respectively; Fig. 2A). The time-course changes of ROS production in both control and MCE-treated hepatocytes are presented in Figure 2B. Even in the control group, DCFH-DA fluorescence intensity increased substantially during the 4-hr incubation period. Nevertheless, the fluorescence intensity in MCE-treated cells was significantly higher than the control group from 1 hr onward. At the end of the test (4 hr), the DCFH-DA fluorescence intensity in the treated cells was about 50% higher than the control (1445 ± 108 vs. 954 ± 55 arbitrary units).

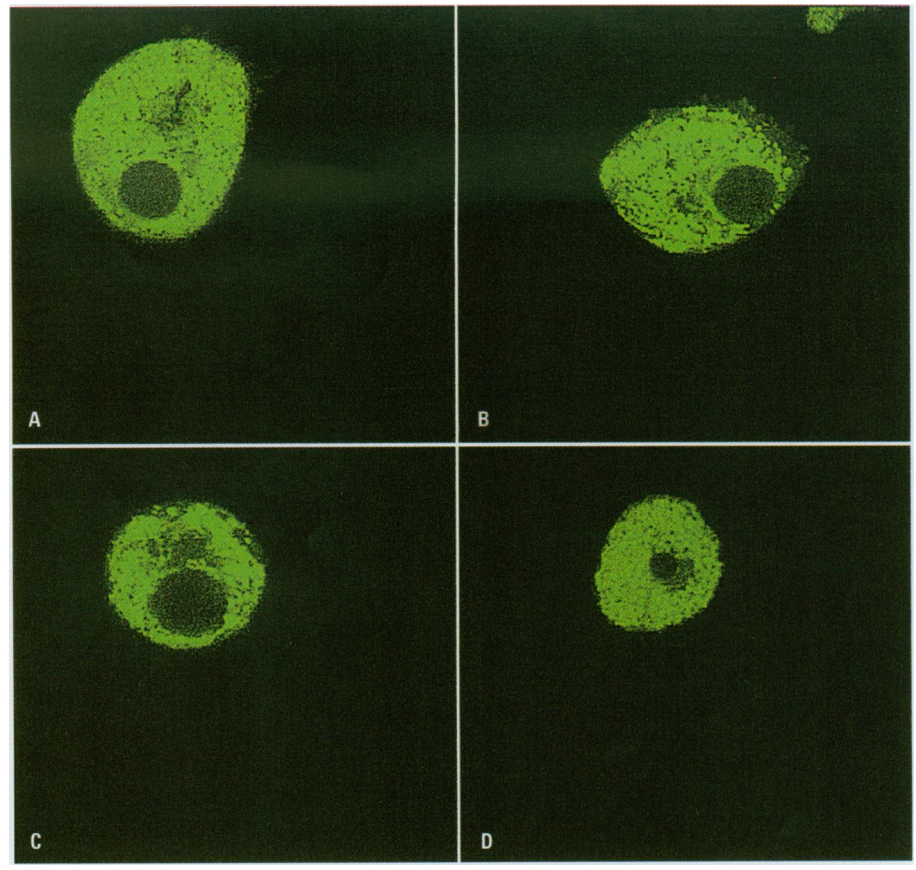

Figure 3. Confocal images of mitochondria in primary cultured rat hepatocytes in the dose–response study. Cells were treated with MCE for 60 min as follows: (A) control, (B) low concentration of MCE, (C) moderate concentration of MCE, and (D) high concentration of MCE. Cells were loaded with Rh-123 (6 μg/ml) and propidium iodide (1.5 μM) for 30 min before analysis. No evident nuclear staining of propidium iodide was observed in any group.

MCE-induced MMP Changes in Primary Cultured Rat Hepatocytes

Using the confocal laser scanning imaging system, the fluorescence of Rh-123 was visualized in the cytosol of hepatocytes, and the cytoplasmic structures stained with Rh-123 appeared to be typical of mitochondria (Figs. 3 and 5). Figure 3 shows the confocal images of mitochondria when cells were treated for 60 min with low, moderate, and high concentrations of MCE. The corresponding changes of fluorescence intensity of Rh-123 were measured and are presented in Figure 4. At the low concentration of MCE (equivalent to 1.25 μg lyophilized algae cells/ml), a slight increase of Rh-123 fluorescence was noted. However, when cells were treated with moderate and high concentrations of MCE (equivalent to 12.5 and 125 μg lyophilized algae cells/ml, respectively), the fluorescence intensity of mitochondria decreased sharply, indicating the release of Rh-123 from mitochondria into the cytosol. No obvious PI staining of nuclei was observed in any of the three treated groups.

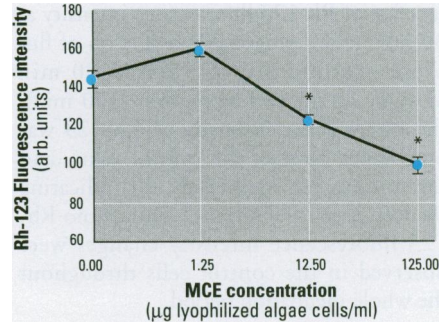

Figure 4. Corresponding changes of mitochondrial Rh-123 fluorescence intensity from the dose–response study. Data are means ± standard deviations from three different measurements and were analyzed using one-way ANOVA with Scheffe's test. *$p<0.05$ compared to the control group (0 concentration).

In the time-course study, hepatocytes were exposed to a concentration of MCE (equivalent to 125 μg lyophilized algae cells/ml) up to 120 min, and the results are shown in Figures 5 and 6 for confocal images and Rh-123 fluorescence intensity, respectively. The data showed an initial

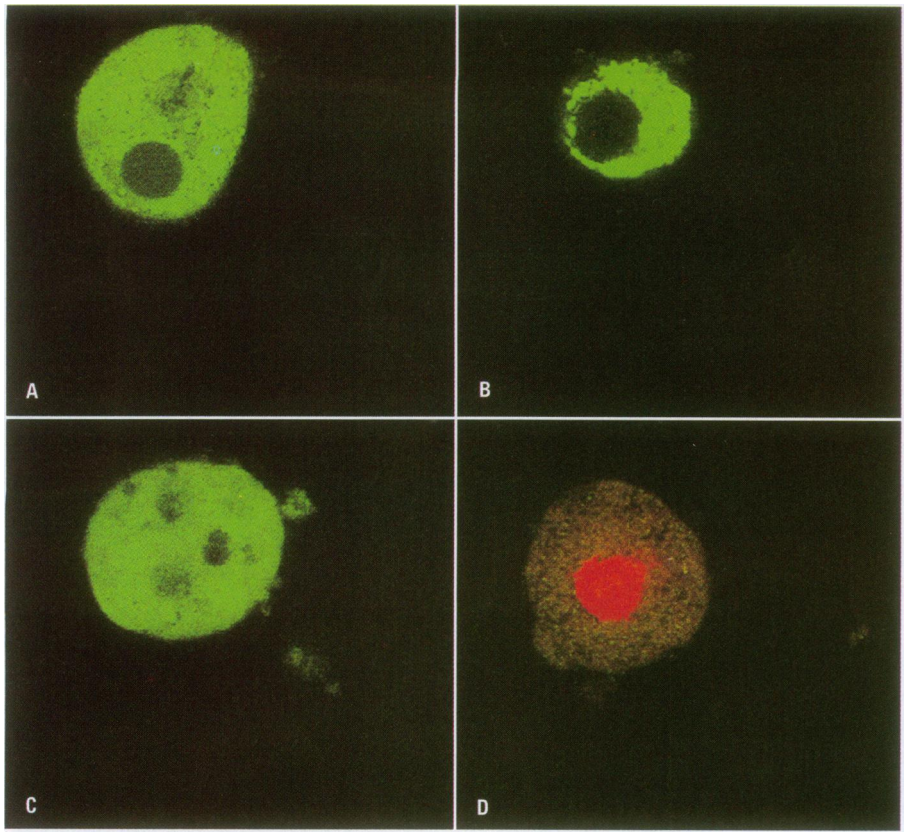

Figure 5. Confocal images of mitochondria in primary cultured rat hepatocytes in the time-course study. Cells were treated with a high concentration of MCE (equivalent to 125 µg lyophilized algae cells/ml) for (A) 0 min, (B) 30 min, (C) 60 min, and (D) 120 min. Cells were loaded with Rh-123 (6 µg/ml) and propidium iodide (1.5 µM) for 30 min before analysis. Obvious nuclear staining of propidium iodide was only noted in cells treated with MCE for 120 min.

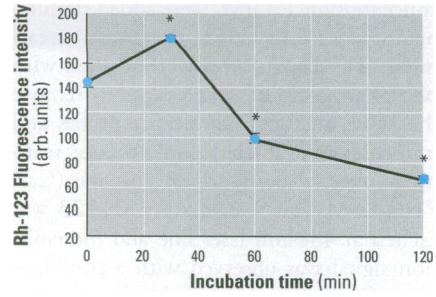

Figure 6. Corresponding changes of mitochondrial Rh-123 fluorescence intensity from the time-course study. Data are expressed as means ± standard deviations from three different measurements and were analyzed using one-way ANOVA with Scheffe's test. *$p<0.05$ compared to the control group (0 concentration).

increase of Rh-123 fluorescence intensity at 30 min, and a progressive reduction of fluorescence intensity was found 30 min onward. At the end of the test (120 min), the fluorescence intensity of Rh-123 was only about 50% of the control value, and the nucleus was stained by PI, indicating the loss of cell viability. In contrast, no Rh-123 fluorescence intensity changes were observed in the control cells throughout the whole incubation period.

Discussion

Mitochondria are known to be vulnerable targets of various toxins because of their important role in maintaining cellular structures and functions. The functional alterations of mitochondria are usually manifested by the changes in MMP. In recent years, using Rh-123 to study MMP changes has become a valuable tool in assessing mitochondrial damage (*18,19*). Rh-123 is a cationic fluorescent probe which accumulates electrophoretically in the strongly negatively charged matrix of mitochondria, and the mitochondrial Rh-123 fluorescent intensity changes (*17*). In the present study, the involvement of mitochondrial damage in the hepatotoxicity of cyanobacteria was demonstrated by the dose- and time-dependent changes of Rh-123 fluorescence intensity in primary cultured rat hepatocytes exposed to MCE (Figs. 3–6). These findings are basically consistent with earlier studies showing the functional and morphological mitochondrial alterations caused by microcystins (*13–16*). At present, there is no clear evidence to suggest the exact mechanism for the adverse effects of cyanobacteria or microcystins on mitochondria. Some earlier studies indicated that ATP depletion might be involved in this process (*14,24*). For instance, Pace et al. (*14*) found that after the rat liver was perfused with microcystin-LR, one of the major toxic compounds produced by cyanobacteria, the isolated mitochondria displayed more than 50% inhibition of cellular respiration.

In the present study, an elevated level of intracellular ROS formation was noted in primary cultured rat hepatocytes exposed to MCE (Fig. 2). More interestingly, marked increase of ROS formation occurred after 1 hr of treatment, coinciding with the temporal changes of MMP in MCE-treated hepatocytes (Figs. 5 and 6). In our earlier study, MCE-induced ROS formation and cellular injury were inhibited by deferroxamine, an antioxidant and specific iron chelator, suggesting the implication of oxidative stress in the hepatotoxicity of MCE (*7*). Mitochondria are well known to be the principal source of intracellular ROS production, and the enhanced level of ROS generation is one of the direct causes of oxidative damage (*11*). It is thus possible that mitochondria are one of the target organelles of oxidative stress caused by microcystins or cyanobacteria.

One of the main toxic mechanisms for microcystins is to act as potent inhibitors of protein phosphatases 1 and 2A, resulting in an increased level of protein phosphorylation, which has a direct impact on the cytoskeleton (*6*). The hyperphosphorylated cytoskeletal proteins cause destruction of structure and homeostatic integrity, manifested by morphological changes and cytotoxicity (*5,25,26*). In the present study, the cytotoxic effects of MCE were assessed by staining of nuclei by PI and by morphological changes examined under light microscope. The results demonstrated that nuclear staining with PI, an indicator for the loss of cell viability, was only found at 2 hr after treatment, following the decrease of Rh-123 fluorescence intensity (Fig. 3A). This finding was similar to the results observed by Zahrebelski et al. (*27*) in their study of chemical hypoxia in cultured rat hepatocytes. Obvious morphological changes, observed by light microscopy, were noted from 3 hr onward, and the typical changes were recorded with 12 hr of incubation (Fig. 1). These morphological changes occurred later than the MMP changes and the staining of nuclei by PI. Therefore, the preceding changes of MMP and ROS formation before cell death are consistent with the notion that mitochondrial damage is closely associated with cellular injury caused by cyanobacteria.

In summary, the dose- and time-dependent changes of Rh-123 fluorescence intensity in MCE-treated hepatocytes indicate that mitochondria damage is an important event in the hepatotoxicity caused by cyanobacteria extract. The exact relationship between mitochondrial damage, ROS formation, and oxidative stress in MCE-treated hepatocytes remains to be further investigated.

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