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

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Microcystic 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 microcystins 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, Heps 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 16,000g 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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fraction was then passed through a preconditioned Sep-Pak C18 cartridge (3-ml tube, Supelco, Bellefonte, 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 $4 \times 10^6$ 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. 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 $1 \times 10^5$ 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 μg 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 6 μg/ml) were added 15 min before Rh-123 and PI, respectively.
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 dichroic 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).

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.

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
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. Mitochondrial Rh-123 fluorescent probe can be accumulated electrochemically in the strongly negatively charged matrix of mitochondria, and the mitochondrial Rh-123 fluorescent intensity changes. 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. These findings are basically consistent with earlier studies showing the functional and morphological mitochondrial alterations caused by microcystins. 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. For instance, 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. More interestingly, marked increase of ROS formation occurred after 1 hr of treatment, coinciding with the temporal changes of MMP in MCE-treated hepatocytes. In our earlier study, MCE-induced ROS formation and cellular injury were inhibited by deferoxamine, an antioxidant and specific iron chelator, suggesting the implication of oxidative stress in the hepatotoxicity of MCE. Mitochondria are well known to be the principal source of intracellular ROS production, and the enhanced level of ROS generation in one of the direct causes of oxidative damage. It is thus possible that mitochondria are one of the major 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. The hyperphosphorylated cytoskeletal proteins cause destruction of structure and homeostatic integrity, manifested by morphological changes and cytotoxicity. 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. This finding was similar to the results observed by 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. 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.

References and Notes

1. Carmichael WW. The toxins of cyanobacteria. Sci Am 270:78-86 (1994).
2. Reesam R, Soong FS, Fitzgerald J, Turczynowicz L, ElSaad O, Roder D, Maynard T, Falconer IR. Health Effects of Toxic Cyanobacteria (Blue-green Algae). Canberra: Australian Government Publishing Service, 1994.
3. Miura GA, Robinson NA, Geibert TW, Bostian KA, White JD, Pace JG. Comparison of in vivo and in vitro toxic effects of microcystin-LR in fasted rats. Toxicon 27:1229-1240 (1991).
4. Miura GA, Robinson NA, Geibert TW, Bostian KA, White JD, Pace JG. Comparison of in vivo and in vitro toxic effects of microcystin-LR in fasted rats. Toxicon 27:1229-1240 (1991).
5. Nishiwaki MR, Ohta T, Nishiwaki S, Suganuma M, Kohyama K, Ishikawa T, Carmichael WW, Fujiki H. Liver tumor promotion by the cyanobacterial cyclic peptide toxin microcystin-LR. J Cancer Res Clin Oncol 118:420-424 (1992).
6. Carmichael WW. Cyanobacteria secondary metabolites—the cyanotoxins. J Appl Bacteriol 72:445-459 (1992).
7. Ding WX, Shen HM, Zhu HG, Ong CN. Studies on oxidative damage induced by cyanobacteria-extract in primary cultured rat hepatocytes. Environ Res (in press).
8. Hermansky SJ, Stoths SJ, Eldeen ZM, Roche VF, Mereish KA. Evaluation of potential chemoprotectors against microcystin-LR hepatotoxicity in mice. J Appl Toxicol 11:62-72 (1991).
9. Mereish KA, Solow R. Effects of antihypertoxic agents against microcystin-LR toxicity in cultured rat hepatocytes. Pharm Res 7:256-259 (1990).
10. Mereish KA, Bunner DL, Regland DR, Creasia DA. Protection against microcystin-LR induced hepatotoxicity by Silymarin: biochemistry, histopathology, and lethality. Pharm Res 8:273-277 (1991).
11. Ames BN, Shimangaka M, Hagen TM. Oxidants, antioxidants, and the degenerative diseases of aging. Proc Natl Acad Sci USA 90:7915-7922 (1993).
12. Papa S. Mitochondrial oxidative phosphorylation changes in the life span. Molecular aspects and physiopathological implications. Biochem Biophys Acta 1276:97-105 (1996).
13. Bhattacharya R, Rao PV, Bhaskar AS, Pant SC, Dube SN. Liver slice culture for assessing the hepatotoxicity of freshwater cyanobacteria. Hum Exp Toxicol 15:105-110 (1996).
14. Pace JG, Robinson NA, Miura GA, Matson CF, Geibert TW, White JD. Toxicity and kinetics of [3H]microcystin-LR in isolated perfused rat livers. Toxicol Appl Pharmacol 107:391-401 (1991).
15. Khan SA, Ghose S, Wickstrom M, Miller LA, Hass R, Haschem WK, Beasley VR. Comparative pathology of microcystin-LR in cultured hepatocytes, fibroblasts, and renal epithelial cells. Nat Toxins 3:119-128 (1995).
16. Wickstrom M, Haschem W, Henningens G, Miller LA, Wyman J, Beasley V. Sequential ultrastructural and biochemical changes induced by microcystin-LR in isolated perfused rat livers. Nat Toxins 4:189-205 (1996).
17. Chen LB. Mitochondrial membrane potential in living cells. Annu Rev Cell Biol 4:155-181 (1996).
18. Rahn CA, Bombick DW, Doolittle DJ. Assessment of mitochondrial membrane potential as an indicator of cytotoxicity. Fundam Appl Toxicol 16:426-448 (1991).
19. Ubl JJ, Chatton JY, Chen S, Stucki JW. A critical evaluation of in situ measurement of mitochondrial electrical potentials in single hepatocytes. Biochem Biophys Acta 1276:124-132 (1996).
20. Harada K, Suzuki M, Dahlem AM, Beasley VR, Carmichael WW, Rinchart KL. Improved method for purification of toxic peptides produced by cyanobacteria. Toxicon 28:435-439 (1988).
21. Shen HM, Ong CN, Shi CY. Involvement of reactive oxygen species in aflatoxin B1-induced cell injury in cultured rat hepatocytes. Toxicology 99:115-123 (1995).
22. Shen HM, Shi CY, Shen Y, Ong CN. Detection of elevated reactive oxygen species level in cultured rat hepatocytes treated with aflatoxin B1. Free Radical Biol Med 21:139-146 (1996).
23. Palmeria CM, Moreno AJM, Madeira VMC, Wallace KB. Continuous monitoring of mitochondrial membrane potential in hepatocyte cell suspensions. J Pharmacol Toxicol Methods 25:35-43 (1996).
24. Wu EY, Smith MT, Bellomo G, Di-Monte D. Relationships between the mitochondrial transmembrane potential, ATP concentration, and cytotoxicity in isolated rat hepatocytes. Arch Biochem Biophys 292:359-362 (1990).
25. Hooser SB, Beasley VR, Lovell RA, Carmichael WW, Haschem WK. Toxicity of microcystin LR, a cyclic hepatopptide hepatotoxic from Microcystis aeruginosa, to rats and mice. Vet Pathol 26:246-252 (1989).
26. Eriksson JE, Toivola D, Meriluoto JA, Karaki H, Han YG, Hartshorne D. Hepatocyte deformation induced by cyanobacterial toxins reflects inhibition of protein phosphorylation. Biochem Biophys Res Commun 173:1347-1353 (1990).
27. Zahrebelski G, Nieminen AL, al-Ghoul K, Qian T, Herman B, Lemasters JJ. Progression of subcellular changes during chemical hypoxia to cultured rat hepatocytes: a laser scanning confocal microscopic study. Hepatology 21:1361-1372 (1995).

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