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Responses of antioxidant systems in the hepatocytes of common carp (*Cyprinus carpio* L.) to the toxicity of microcystin-LR

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Abstract

The freshwater, bloom-forming cyanobacterium (blue-green alga) *Microcystis aeruginosa* produces a peptide hepatotoxin, which causes the damage of animal liver. Recently, toxic *Microcystis* blooms frequently occur in the eutrophic Dianchi Lake (300 km^2 and located in the South-Western of China). Microcystin-LR from *Microcystis* in Dianchi was isolated and purified by high performance liquid chromatography (HPLC) and its toxicity to mouse and fish liver was studied (Li et al., 2001). In this study, six biochemical parameters (reactive oxygen species, glutathione, superoxide dismutase, catalase, glutathione peroxide and glutathione S-transferase) were determined in common carp hepatocytes when the cells were exposed to 10 μg microcystin-LR per litre. The results showed that reactive oxygen species (ROS) contents increased by more than one-time compared with the control after 6 h exposure to the toxin. In contrast, glutathione (GSH) levels in the hepatocytes exposed to microcystin-LR decreased by 47% compared with the control. The activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxide (GSH-Px) increased significantly after 6 h exposure to microcystin-LR, but glutathione S-transferase (GST) activity showed no difference from the control. These results suggested that the toxicity of microcystin-LR caused the increase of ROS contents and the depletion of GSH in hepatocytes exposed to the toxin and these changes led to oxidant shock in hepatocytes. Increases of SOD, CAT and GSH-Px activities revealed that these three kinds of antioxidant enzymes might play important roles in eliminating the excessive ROS. This paper also examined the possible toxicity mechanism of microcystin-LR on the fish hepatocytes and the results were similar to those with mouse hepatocytes.

Keywords: Microcystin-LR; Antioxidant system; Common carp; Hepatocyte; Glutathione; Superoxide dismutase; Catalase; Glutathione peroxide; Glutathione S-transferase; Reactive oxygen species

1. Introduction

Recently, *Microcystis* bloom frequently occurred in the eutrophic Dianchi Lake and the dominant species is *Microcystis aeruginosa*, which produces a family of related cyclic heptapeptides (microcystins, MC) (Botes et al., 1985). These toxins are severely hepatotoxic (Carmichael et al., 1985), are produced in *Microcystis* cells and are released into water body when algal cells are broken. Degradation of MC in water is slow and the toxins often remain in the water supply used by people. There are many reports that *Microcystis* bloom leads to liver damage in human populations whose water supplies were contaminated by toxic *Microcystis* (Falconer et al., 1983; Carmichael et al., 1985; Carmichael, 1994; Bell and Codd, 1994). Therefore, microcystins remaining in water supply become a severe threat to human health.

Studies on toxicity of microcystin-LR on animal hepatocytic antioxidant systems have been carried out and the results demonstrated that antioxidant systems (mainly

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GSH) could be relevant indices in explaining the sensitivity of some vertebral species to MC (Runnegar et al., 1987; Takenaka and Otsu, 1999). But little is known about the responses of antioxidant enzymes activities to microcystin-LR in freshwater fish hepatocytes and the relationship between ROS contents and MC shock. In this study, antioxidant enzymatic activities (superoxide dismutase, catalase, glutathione peroxide and glutathione S-transferase), glutathione levels and reactive oxygen species (ROS) contents were measured in the common carp hepatocytes exposed to 10 μg/l microcystin-LR.

2. Materials and methods

2.1. Toxin and reagents

MC-LR was isolated and purified with the improved HPLC method (Harada et al., 1988). The toxin was first dissolved in a small amount of sterile phosphate-buffered saline (pH 7.2) for storage and was diluted with the same buffer saline to the needed concentration before tests began. Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum, penicillin and streptomycin were obtained from GIBCO BRL (Grand Island, NY 14072, USA). Dihydrorhodamine 123, NADH and glutathione were purchased from Sigma (St Louis, MO, USA). Other reagents, obtained from various commercial sources, were analytical or higher grades.

2.2. Hepatocyte isolation

Hepatocytes were isolated from carp (1000 g, female) with 0.25% trypsin digestion at 25 °C for 40 min. Cells were suspended in DMEM and washed with the same medium. Isolated hepatocytes were cultured in DMEM supplied with penicillin (100 IU/ml), streptomycin (100 μg/ml), and 5% fetal bovine serum. Five milliliter cell suspension were seeded into 25 ml cell culture bottle at a concentration of 1×10^6 cells/ml and incubated at 25 °C free from CO_2. Cell viability was assessed with the trypan blue exclusion method. The number of cells in suspension was determined in a Neubauer counting Chamber and 150 cells were evaluated per sample.

2.3. MC-LR exposure

The MC-LR stock solution was added directly to the medium with hepatocytes just isolated from carp liver for toxin exposure, the finial toxin concentration in the cultures was made to be 10 μg/l and the cells were cultured in the same way as above. Hepatocytes were collected at the time intervals of 15 min, 0.5, 1, 2, 4 and 6 h, respectively, after exposure and centrifuged at 500*g* for 15 min. The cells obtained after centrifugation were homogenized to a 1/2 (w/v) ratio in sterile phosphate-buffered saline (pH 7.2). The supernatants obtained after centrifugation at 4000*g* were stored at − 70 °C for assay. All these steps were carried out at 4 °C.

2.4. ROS contents and GSH levels assays

Intracellular ROS formation was analyzed by the method of Winzer et al. (2001) who assayed intracellular ROS formation with dihydrorhodamine 123. Fluorescence was measured by excitation at 450–490 nm and emission at >520 nm using an image analysis system. Total glutathione was assayed by the method of Griffith (1980). All the experiments were carried out in triplicate.

2.5. Antioxidant enzyme activities assays

SOD activity was measured according to the method of Paoletti et al. (1986) based on the inhibitory action of the enzyme on the rate of NADH oxidation, and catalase activity was determined by the method of Beers and Sizer (1952). Glutathione peroxide and glutathione S-transferase activities were assayed by the kits supplied by The Nanjing Bioengineering Institute, China. All the experiments were carried out in triplicate.

2.6. Statistics

All data were evaluated by one-way ANOVA (Spss 6.0.1 for windows, tests: least significant difference, Tukey's honestly significant difference).

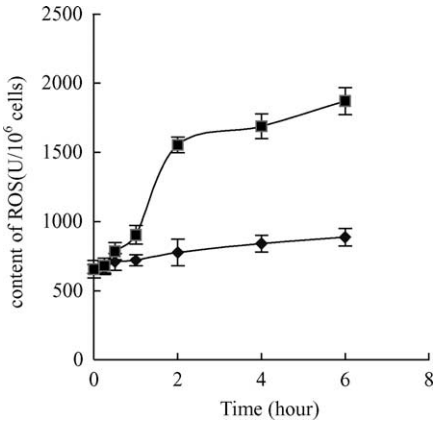

Fig. 1. The changes of ROS contents in carp hepatocytes after 6 h exposure to 10 μg/l MC-LR. Within 1–6 h exposure, ROS contents in the treated cells were significantly different from controls ($P < 0.05$). Vertical bars show standard deviation of each data set. ROS, reactive oxygen species; MC-LR, microcystin-LR; ((■) treatment, (◆) control).

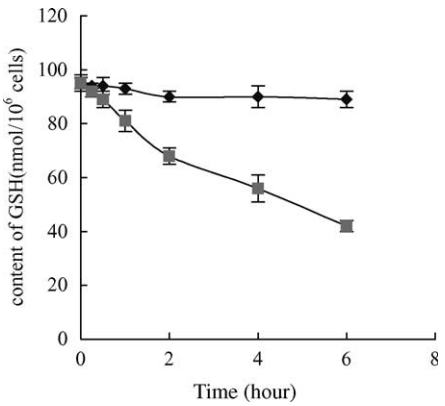

Fig. 2. Response of GSH levels in carp hepatocytes after 6 h exposure to 10 μg/l MC-LR. Within 0.5–6 h exposure, GSH contents in the treated cells were significantly different from controls ($P < 0.05$). Vertical bars show standard deviation of each data set. GSH, reduced glutathione; ((■) treatment, (◆) control).

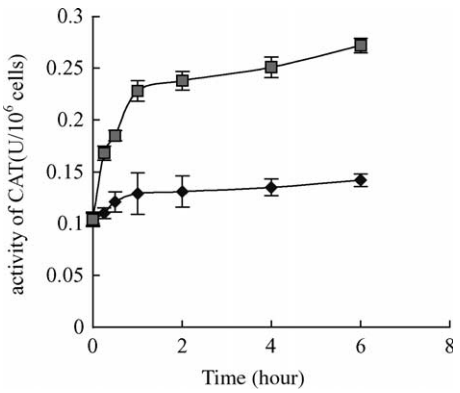

Fig. 4. Response of CAT activity in carp hepatocytes after 6 h exposure to 10 μg/l MC-LR. Within 0.25–6 h exposure, CAT activities of the treated cells were significantly different from controls ($P < 0.05$). Vertical bars show standard deviation of each data set. CAT, catalase; ((■) treatment, (◆) control).

3. Results

3.1. Formation of intracellular ROS and change of GSH level

Significant induction of intracellular ROS formation was found in hepatocytes of the common carp after 0.5 h of exposure to 10 μg/l MC-LR and ROS contents increased rapidly within 2 h (Fig. 1). The increase in ROS level of the treated hepatocytes was almost two-times higher than the control after 6 h exposure. In contrast to these changes, GSH levels decreased after 15 min exposure to the toxin and hepatocytes exhibited depletion of GSH after 6 h exposure (Fig. 2).

3.2. Antioxidant enzyme activities changes

SOD activity increased obviously after 6 h exposure to MC-LR compared with the control ($P < 0.05$) (Fig. 3) and the catalase activity change was similar to SOD when the cells were treated with 10 μg/l MC-LR (Fig. 4). The increase in glutathione peroxide activity could be registered within 0.5 h exposure, but decreased sharply after 0.5 h exposure and then in the same way as the control (Fig. 5). However, only glutathione S-transferase activity exhibited

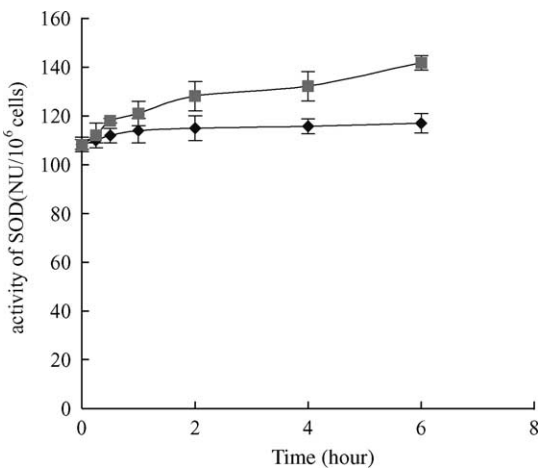

Fig. 3. SOD activity increase after 6 h exposure to 10 μg/l MC-LR. Within 1–6 h exposure, SOD contents in the treated cells were significantly different from controls ($P < 0.05$). Vertical bars show standard deviation of each data set. SOD, superoxide dismutase; ((■) treatment, (◆) control).

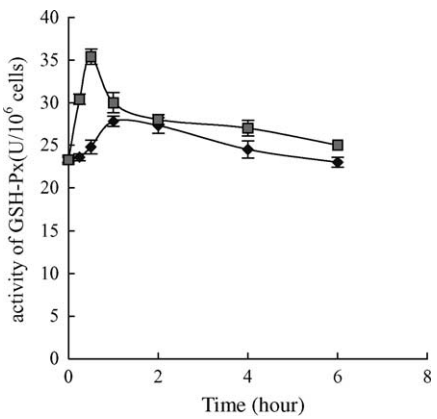

Fig. 5. The change of GSH-Px activity after 6 h exposure to 10 μg/L MC-LR. GSH-Px activities of the treated cells were significantly different from controls ($P < 0.05$) within 1 h exposure. Vertical bars show standard deviation of each data set. GSH-Px, glutathione peroxide; ((■) treatment, (◆) control).

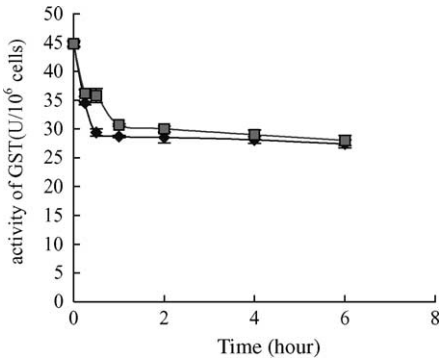

Fig. 6. The decrease in GST activity after 6 h exposure to 10 μg/l MC-LR. No difference between the treatment and the control was found after 6 h exposure. Vertical bars show standard deviation of each data set. GST, glutathione S-transferase; ((■) treatment, (◆) control).

a decline after exposure, showing no difference with the control (Fig. 6).

4. Discussion

In our study, a obvious increase of hepatocytic ROS contents was observed after 15 min exposure to 10 μg/l MC-L compared with control and this change remained the same within 6 h. ROS increase suggested that the hepatocytes were under oxidative stress as the result of exposure to the toxin. Considering the crucial role of GSH in cellular antioxidant protection, a marked decrease of GSH contents in the treated cells indicated that GSH takes part in eliminating cytosolic ROS. Meanwhile, the activity increases of antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxide) also reflected that these enzymes played important roles in clearing away excessive ROS and regenerating reduced glutathione. These results were consistent with the previous MC toxicological studies on rate and fish, respectively (Runnear et al., 1987; Takenaka and Otsu, 1999).

No change of GST activity was recorded during 6 h exposure to MC-LR and this kind of enzyme appeared less interesting biomarker than the above parameters for short term exposure to the toxin.

From the present study, we could infer the possible toxicity mechanism of microcystin-LR on the fish hepatocytes. MC can potently inhibit protein phosphatase type-1 and 2A after the toxin was transported to cytoplasm by the bile acid transporter in the cell membrane of hepatocytes and the inhibition may disturb the cellular phosphorylation balance (Yoshizawa et al., 1990; Matsuhima et al., 1990; Eriksson et al., 1990a; Runnegar et al., 1991, 1999), caused the marked increase of ROS contents and the depletion of GSH in hepatocytes. As a result, these changes would lead to oxidant shock in hepatocytes. Although antioxidant enzymes (SOD, CAT and GSH-Px) had played their roles in eliminating cytosolic ROS and regenerating GSH, they could not prevail over the oxidant stress induced by the toxin. Therefore, this effect caused damage of hepatocytes, leading to apoptosis and even necrosis of cells.

Acknowledgements

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