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Endoplasmic reticulum stress in murine liver and kidney exposed to microcystin-LR

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ABSTRACT

To investigate the effect of microcystin-LR (MC-LR) on apoptosis based on the endoplasmic reticulum stress (ERS) pathway in mouse liver and kidney, male ICR mice were intraperitoneally injected with 20 μg kg^{-1} body weight MC-LR for 21 days, and mRNA and protein levels of ERS special molecules in liver and kidney were analyzed using quantitative real-time PCR and western blotting. MC-LR significantly improved mRNA and protein expression of C/EBP homologous protein (CHOP) and cleaved caspase-12 in liver, whereas it inhibited expression of CHOP and caspase-12 in kidney. MC-LR also induced significant down-regulation of B-cell lymphoma/leukemia-2 (Bcl-2) mRNA expression in liver and weak up-regulation in kidney. These results indicated the involvement of the ERS pathway in MC-LR-induced apoptosis of hepatic cells but not in renal cells of mice. The weight changes and histological damage of liver and kidney were in accordance with the appearance of ERS. Our results indicate that ERS plays an important role in hepatic cell apoptosis induced by MC-LR, and is considered as a new pathway of liver toxicity. Its relative special genes might be considered as potentially new biomarkers used for risk assessment of MC-LR in the environment.

1. Introduction

Cyanobacteria are widely distributed in terrestrial and aquatic environments around worldwide, and produce a range of toxins that are associated with adverse health effects in animals and humans (Codd et al., 2005; Ince and Codd, 2005). Microcystins are a family of cyclic heptapeptides that are produced by cyanobacteria, and they are considered to be potential inhibitors of protein phosphatase type 1 and 2A (PP1 and 2A), as well as skin and liver tumor promoters in animals (Nishiwaki-Matsushima et al., 1992). Epidemiological investigations also have demonstrated that microcystins cause stomach and intestinal inflammation, liver cancer, and disease of the spleen in humans (Yu, 1996; Ueno et al., 1996). Microcystin-LR (MC-LR) is one of the most commonly studied and most harmful microcystins, and it is produced by cyanobacteria, e.g. *Microcystis* spp. (Hoeger et al., 2005). L and R represent leucine and arginine on the molecular structure, respectively (Fastner et al., 2002).

As an important environmental contaminant, MC-LR has been found to induce apoptosis in several types of cells, including hepatocytes of rats (Sturgeon and Towner, 1999) and humans (Chen et al., 2006). MC-LR is also toxic to the kidney (La-Salete et al., 2008), and it promotes renal alterations, affects renal physiology (Nobre et al., 2003) and induces renal cell apoptosis (Komatsu et al., 2007). Mice exposed to sublethal doses (20, 30 and 45 μg kg^{-1} d^{-1}) of MC-LR for 7 days exhibit high levels of apoptotic cells in the

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centrilobular and perinecrotic regions of the liver (Yoshida et al., 1998). Although it has been demonstrated that MC-LR can induce hepatic cell apoptosis through the mitochondrial and reactive oxygen species (ROS)-dependent oxidative stress pathways (Botha et al., 2004; Wei et al., 2008), the underlying mechanism has not been fully elucidated. Recently, more concern has been focused on the involvement of endoplasmic reticulum stress (ERS) in apoptosis. For example, genistein has been found to induce apoptosis in human hepatocellular carcinoma through ERS (Chiang et al., 2005).

The ER is one of the largest cell organelles and comprises a network of interconnected and closed membrane-bound vesicles, in which protein molecules are assembled (Ron and Walter, 2007). Correctly folded proteins can be transported out of the ER, whereas incompletely folded proteins are retained in the organelle to complete the folding process or to be targeted for destruction. A number of environmental changes, such as various toxic insults, can disturb ER functions and result in ERS (Szegezdi et al., 2006). Under ERS, unfolded or misfolded proteins accumulate in the ER lumen, which leads to the unfolded protein response (UPR) (Rutkowski and Kaufman, 2004). UPR is one of the apoptotic pathways that are mediated by ERS. In response to ERS–UPR, ER chaperones, such as the expression of 78-kDa glucose-regulated protein (GRP78) is up-regulated to stabilize protein folding (Kaufman, 1999). Temperate ERS can relieve cellular dysfunction and increase the possibility for survival, but prolonged and/or serious stress leads to apoptosis, which is mediated by transcriptional induction of C/EBP homologous protein (CHOP) (Zinszner et al., 1998; Li and Holbrook, 2004) and/or by a caspase-12-dependent pathway (Momoi, 2004). B-cell lymphoma/leukemia-2 (Bcl-2) gene is distributed in the ER, and it regulates ER homeostasis and cell apoptosis in response to signals that affect ER function (Danial and Korsmeyer, 2004). ERS-mediated apoptosis participates extensively in the apoptotic mechanism of many environmental toxins, such as cigarette (Tagawa et al., 2008), nonylphenol (Gong et al., 2009) and bisphenol A (Tabuchi et al., 2006). However, little information is available about ERS associated with MC-LR exposure.

In the present study, we analyzed the expressions of special genes of ERS–UPR to investigate ERS-mediated hepatic and renal cell apoptosis of mice exposed to MC-LR at 20 $\mu g\ kg^{-1}\ d^{-1}$ for 21 days. The purpose was to clarify the role of ERS involved in MC-LR-induced apoptosis and elucidate the underlying toxicological mechanisms of the ERS pathway in mouse liver and kidney.

2. Materials and methods

2.1. Chemicals

The cyanobacterial toxin MC-LR (purity $\geq$95%, by HPLC) was obtained from Alexis Biochemicals (Lausen, Switzerland). MC-LR was dissolved in the minimum amount of DMSO (Amresco, Solon, OH, USA) (0.1%) and diluted to the required concentration in sterile saline. Final concentration of DMSO in the mouse-exposed MC-LR solution was $<$0.01%. All other chemicals were obtained from Sigma–Aldrich Inc. (St. Louis, MO, USA) unless otherwise mentioned.

2.2. Animals and treatments

Healthy male ICR mice (8 weeks old and 25–30 g in weight) were provided by the Animal Center, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China. The mice were acclimatized for at least 1 week before being divided into MC-LR and control groups. Five mice were housed in each polycarbonate cage on hardwood bedding under a 12 h light/dark cycle with access to water and food *ad libitum*. Room temperature and relative humidity were kept at 21 $\pm$ 0.3 °C and 60 $\pm$ 5%, respectively. In the MC-LR group, mice were injected intraperitoneally daily with MC-LR at a dose of 20 $\mu g\ kg^{-1}$ body weight for 21 days. In the control group, the mice were exposed to 0.9% sterile saline solution. All animal experimental procedures were performed in strict accordance with the care and use of laboratory animals (National Research Council, Washington, DC) and the related ethical regulations of Nanjing University.

2.3. Weights of body, liver and kidney and histological damage of liver and kidney

The initial and final body weights were recorded on days 1 and 21, respectively. All the mice were sacrificed on day 22. The liver and kidneys were removed, washed in normal saline, blotted, weighed (absolute weight) and frozen in liquid nitrogen immediately, and stored at -80 °C for further analysis. The relative organ weight was calculated as a ratio of organ to body weight of the mice.

All animals were subjected to histological examination of liver and kidney according to standard procedures (McManus and Mowry, 1965). Tissue samples were fixed in 10% formalin in PBS, dehydrated in a graded series of alcohols, cleared in xylene, and embedded in paraffin wax. Multiple sections from each block were prepared at 4 μm thick and stained with hematoxylin and eosin (H&E) before observation under an optical microscope (Nikon, Tokyo, Japan).

2.4. mRNA expression assays with quantitative real-time PCR (qPCR)

Total RNA was isolated from liver or kidney tissue by a homogenizer (Retsch, Haan, Germany) with TRIZOL reagent according to the manufacturer's protocol (TaKaRa, Shiga, Japan). About 100 mg frozen liver or kidney tissue was homogenized in 1 ml TRIZOL on ice. Homogenates were transferred into 1.5-ml tubes and 0.2 ml chloroform was added to each tube. After vortexing for 15 s, mixtures were kept at room temperature for 5 min and centrifuged at 12,000$\times g$ for 15 min at 4 °C. Aqueous supernatant was carefully transferred into a new tube without disturbing the bottom phase, and 0.5 ml isopropanol was added and mixed with vortexing. The mixture was kept at room temperature for 10 min and centrifuged at 12,000$\times g$ for 10 min at 4 °C. The pellets were washed with 1 ml 75% ethanol and centrifuged at 7500$\times g$ for 5 min at 4 °C before air-drying for 10 min, and resuspended in RNase-free water.

After RNA concentration was determined by biophotometer (Eppendorf, Hamburg, Germany), the purified total RNA (1 μg) was reverse-transcribed using a first-