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Toxic effects of microcystin-LR on the HepG2 cell line under hypoxic and normoxic conditions

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ABSTRACT: Microcystins (MCs) are highly liver-specific and evidenced as a liver tumor promoter. Oxidative stress is one of the most important toxicity mechanisms of MCs, which is tightly related to oxygen concentration. The effects of MCs on animals and cell lines in normoxia and the mechanisms have been well studied, but such effects in different oxygen conditions were still unclear. The aim of the present study was to explore the cellular response of the human hepatocellular carcinoma cell line (HepG2) to MC-LR exposure under hypoxic (1% O_2) and normoxic (21% O_2) conditions. We examined cell viability, mitochondrial membrane potential (MMP), superoxide dismutase (SOD) activity and gene expression posttoxin exposure. Cell viability was increased by MC-LR in normoxia although decreased in hypoxia. MC-LR markedly induced MMP loss under hypoxic condition but only slightly MMP loss under normoxic condition. SOD activity was significantly induced by MC-LR in hypoxia, indicating prolonged oxidative stress. Inhibitory apoptosis protein (c-IAP2) was significantly up-regulated by MC-LR under normoxic condition, suggesting that c-IAP2 played an important role in the promotion of cell proliferation by MC-LR. These results indicate that MC-LR promotes cell proliferation under normoxic condition whereas induces cell apoptosis under hypoxic condition. **Copyright © 2012 John Wiley & Sons, Ltd.**

Keywords: MC-LR; hypoxia; normoxia; oxidative stress; apoptosis; proliferation

Introduction

Cyanobacterial blooms are a worldwide problem as a result of the production of cyanotoxins (Dawson, 1998). Natural exposure causes a high health risk to aquatic organisms, wild life and humans upon ingesting toxins from cyanobactera or water (Chen *et al*., 2009; Malbrouck and Kestemont, 2006). Microcystins (MCs) are the most commonly found group of cyanotoxins and more than 80 variants are known (Dawson 1998; Fastner *et al*., 2002; Hoeger *et al*., 2005). It has been elucidated that microcystins are highly hepatotoxic and evidenced as a liver tumor promoter (Nishiwaki-Matsushima *et al*., 1992). It is well recognized that MCs are protein phosphatase 1 and 2A potent inhibitors, leading to increased protein phosphorylation, which is directly related to their tumor-promoting activity (Carmichael, 1994; Hooser *et al*., 1989; Hooser, 2000).

Recent studies suggest that oxidative stress is considered as another important mechanism of microcystin toxicity. Previous studies found that MCs could cause oxidative stress by increasing intracellular reactive oxygen species (ROS) production, lipid peroxides and diminishing antioxidant enzymes (Moreno *et al*., 2005; Nong *et al*., 2007; Zegura *et al*., 2004). It has been reported that MC-LR could induce mitochondrial damages and alter Na^+–K^+-ATPase and Ca^{2+}–Mg^{2+}-ATPase activities of mitochondria (Zhao *et al*., 2008). In addition, Ding *et al*. (2000) reported that there was a significant increase in ROS formation before the mitochondrial permeability transition (MPT) onset and mitochondrial membrane potential (MMP) loss. Recently it has been shown that MC-LR can up-regulate p53, bax and caspases3 genes expression (Li *et al*., 2011; Takumi *et al*., 2010; Zhang *et al*., 2011). These data suggest that oxidative stress and mitochondria play an important role in microcystin-induced apoptosis.

Oxidative stress can be induced by disturbing the balance between ROS formation and antioxidants (Turrens, 2003). ROS is used to describe a variety of molecules and free radicals derived from molecular oxygen (Phillips *et al*., 2003). Hypoxia is a key determinant of tissue pathology during tumor development and a common environmental stress that influences signaling pathways and cell functions (Alvarez-Tejado *et al*., 2001). It has been reported that hypoxia can induce apoptosis and protect against apoptosis by causing oxidative stress and activating death or survival signals (Greijer and van der Wall, 2004; Kietzmann and Gorlach, 2005). As predicted that the mitochondrial production of O_2^- increases with oxygen concentration, so the formation of ROS should decrease with hypoxia (Alvarez

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Journal of Applied Toxicology

et al., 2003; Genova *et al*., 2001; Wittenberg and Wittenberg, 1989). Yet, various groups have reported a paradoxical increase in oxidative stress in moderately hypoxic conditions (Guzy *et al*., 2005; Schumacker, 2002; Waypa and Schumacker, 2002).

Hypoxia is a common environmental stress that influences signaling pathways and cell functions (Alvarez-Tejado *et al*., 2001). In recent years, the toxic mechanisms of microcystins have been well studied. However, cellular toxicity by microcystins in different oxygen concentrations has not been elucidated clearly. To our knowledge, this is the first study to examine the toxic effect of MC-LR on cell line in different oxygen conditions. The main purpose of the present study was to compare the cellular response of the HepG2 cell line to MC-LR under hypoxic (1% O_2) and normoxic (21% O_2) conditions and to explore the underlying toxicological mechanisms of MCs under different oxygen levels.

Materials and Methods

Chemicals and Reagents

MC-LR was obtained from Alexis (Switzerland) and dissolved in ddH_2O to 1 mM and stored at −20°C until use. Work solutions were prepared by diluting the purified extracts in Dulbecco's modified Eagle's medium (DMEM; Hyclon, USA). All reagents obtained from various commercial sources were analytical or higher grades.

Cell Culture and Treatment

The human hepatocellular carcinoma cell line (HepG2) was a gift from Dr Xiao (Institute of Hydrobiology, Chinese Academy of Sciences) and was cultured in DMEM with 10% fetal bovine serum, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin (Invitrogen, USA) in a 5% CO_2 humidified incubator at 37°C. For toxin exposure experiments, HepG2 cells were treated with 0.01, 0.1, 1 and 5 μM MC-LR for 3, 6, 12 and 24 h, and then incubated in normoxic (21% O_2) or hypoxic (1%O_2) conditions, respectively. Each treatment was repeated three times.

Cell Viability Assay

Cell viability was evaluated by measuring mitochondrial dehydrogenase activity based on mitochondrial membrane potential (MTT) (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrasodium bromide). Briefly, 2×10^4 cells/well were seeded in a 96-well plate overnight and then treated with various concentrations of MC-LR for 24 h in hypoxia and normoxia, respectively. After incubation, 20 μl MTT (5mg ml^{-1} in DMEM) was added and incubated at 37 °C for 4 h and then the medium was removed and 100 μl of DMSO added. The absorbance was recorded using microplate reader at 570 nm (Thermo, USA).

Mitochondrial Membrane Potential

We measured the changes in MMP using a JC-1 probe that gives a red fluorescence when MMP is high and green fluorescence when MMP is low that occurs in early apoptosis cells. A JC-1 probe (Beyotime, China) was used to measure mitochondrial membrane potential in HepG2 cells. Briefly, cells were seeded in six-well plates overnight and then treated with various concentrations of MC-LR for 24 h in hypoxia and normoxia, respectively. MC-LR containing medium was removed, the cells were then washed with phosphate-buffered saline (PBS), 1 ml of JC-1 working solution was added and incubated in a 5% CO_2 humidified incubator at 37 °C for 20 min, the supernatant was then discarded and washed twice with JC-1 staining buffer. Next, 2 ml of medium per well was added and MMP was monitored using a fluorescence microscope (TE2000-U, Nikon, Japan). The red JC-1 fluorescence was observed at 525 nm excitation (Ex)/590 nm emission (Em) and the green cytoplasmic JC-1 fluorescence was observed at 485 nm Ex/530 nm Em.

Superoxide Dismutase Activity

Superoxide dismutase (SOD) activity was measured using a superoxide dismutase (SOD) test kit (Nanjing Jiancheng Bioengineering Institute, China) and a modified xanthine–oxidase–cytochrome c method as described by McCord and Fridovich (1969). SOD activity was monitored spectrophotometrically at 550 nm using a microplate reader (Thermo, USA).

RNA Extraction and Quantitative Real-time PCR

Total RNA was isolated using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions and quantified by determination at OD260. Reverse transcription was performed with oligo (dT) primer using a First Strand cDNA Synthesis Kit (TOYOBO, Japan) according to the manufacturer's instructions. The sequences of the primers used in real-time PCR were listed in Table 1. A SYBR Green Q-PCR kit (TOYOBO, Japan) was used as the fluorescent dye for real-time quantitative PCR on a Chromo4 96-well reactor with optical caps (MJ Research, Cambridge, MA, USA). Each sample was run in three tubes. After completion of the PCR amplification, data were analyzed with the Option Monitor software Version 2.03 (MJ Research, Cambridge, MA, USA). The relative expression levels of the genes were used in the formula $2^{-(\nabla\nabla Ct)}$.

Cell Lysate Preparation and Western Blot Analysis

Cells were washed with PBS, and then lysed in a buffer containing 50mM Tris–HCl (PH 7.4), 1% NP40, 0.25% Na-deoxycholate, 150mM NaCl, 1mM EDTA (PH 8.0), 1mM NaF, 1mM PMSF, 1mM Na_3VO_4 and protease inhibitor for 1 h on ice. Samples were then centrifuged at 12 000rpm for 10 min at 4 °C and supernatants were collected. Boiled 50μl protein, 50 μl 2 × loading buffer and 5% β-Mercaptoethanol in boiling water for 5min then stored

Table 1. Primer sequences of bax, p53, caspase3 and gapdh

Gene name		Primer sequences
bax	Forward	5′-CCTGTGCACCAAGGTGCCGGAACT-3′
	Reverse	5′-CCACCCTGGTCTTGGATCCAGCCC-3′
p53	Forward	5′-CTGAGGTTGGCTCTGACTGTACCAC-CATCC-3′
	Reverse	5′-CTCATTCAGCTCTCGGAACATCTC-GAAGCG-3′
caspase3	Forward	5′-TCATTATTCAGGCCTGCCGTGGTA-3′
	Reverse	5′-TGGATGAACCAGGAGCCATCCTTT-3′
gapdh	Forward	5′-TGGACCTGACCTGCCGTCTAG-3′
	Reverse	5′-AGTGGGTGTCGCTGTTGAAGTC-3′

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at −80 °C. For western blot, proteins (20 μg) were loaded on a SDS-polyacrylamide gel, separated by electrophoresis and then electroblotted onto PVDF membranes (Millipore, Germany). Membranes were blocked in TBST containing 5% nonfat milk for 1 h at room temperature and then incubated with anti-c-IAP2 (Santa Cruz, MA, USA; 1: 500) and anti-GAPDH (Santa Cruz, CA, USA; 1: 1000) for 1 h at room temperature. The membranes were then washed in TBST for 10 min, repeated three times and incubated with HRP-conjugated secondary antibody (Pierce, USA; 1: 5000) for 1 h at room temperature. The membranes were then washed with TBS four times for 5 min and the membrane was revealed with chemiluminescent substrates (Pierce, Rockford, IL, USA).

Statistical Analysis

Three independent experiments were conducted for all analyzes. Values were shown as the mean ± standard deviation (SD) and analyzed using one-way ANOVA and Turkey's multiple comparison tests. The differences were considered significant if $P < 0.05$.

Results

Cell Viability

The HepG2 cell viability was determined using a MTT assay (Fig.1). Under hypoxic conditions, cell viability was decreased by 0.1, 1 and 5 μM MC-LR after 24 h of exposure. HepG2 cell viability under normoxic condition was significantly increased at all MC-LR concentrations after 24 h of exposure. Cell viability in the control groups was significantly increased by hypoxia compared with the control in normoxia.

MMP Changes

Figure 2 shows the MMP changes in HepG2 cells under hypoxic (Fig. 2A–E) and normoxic (Fig. 2F–J) conditions. MMPs were lower in hypoxia than in normoxia, and the MMP decrease induced by MC-LR presented a dose–dependent manner under hypoxic and normoxic conditions.

SOD Activity

SOD activity was significantly affected by MC-LR and hypoxia (Fig. 3). In hypoxic conditions (Fig. 3A), SOD activity of the HepG2 cells was much lower than that in normoxic conditions (Fig. 3B) at 3, 12 and 24 h, but increased to the maximum value after 6 h. SOD activity in hypoxia was markedly elevated by all concentrations of MC-LR except for 0.1 μM at 12 h. Under normoxic conditions, 0.01μM MC-LR slightly decreased SOD activity at 6 h, and then increased after 12 and 24 h. SOD activity was significantly increased by 0.1 μM MC-LR at 3 h, and returned to the control level after 6 and 12 h, but decreased to the minimum value at 24 h. Next, 1 μM MC-LR decreased SOD activity after 3 and 12 h exposure and increased SOD activity to the maximum value at 6 h, and then returned to the original level at 24 h. The highest MC-LR dose (5μ M) significantly decreased SOD activity at 3, 6 and 12 h, but recovered to the control level after 24 h.

Quantitative Real-time PCR

The transcriptional changes of bax, p53 and caspase3 are shown in Fig. 4. The expression of bax was significantly up-regulated by MC-LR in both hypoxia (Fig. 4A) and normoxia (Fig. 4B). The p53 transcription was markedly induced at 24 h by 0.1, 1 and 5 μM

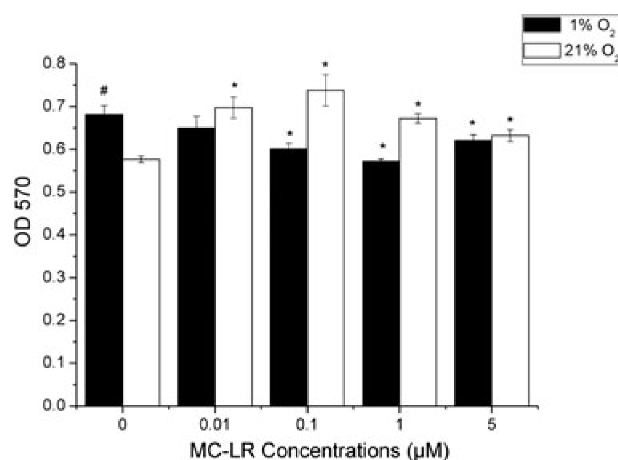

Figure 1. Cell viability of HepG2 cells incubated with MC-LR under different oxygen concentrations. The black and white bars represent hypoxia (1% O_2) and noemoxia (21% O_2), respectively. The values are expressed as mean ± standard deviation (SD). *indicates significant differences at $P < 0.05$ and **indicates significant change at $P < 0.01$ in comparison with determined control group values.

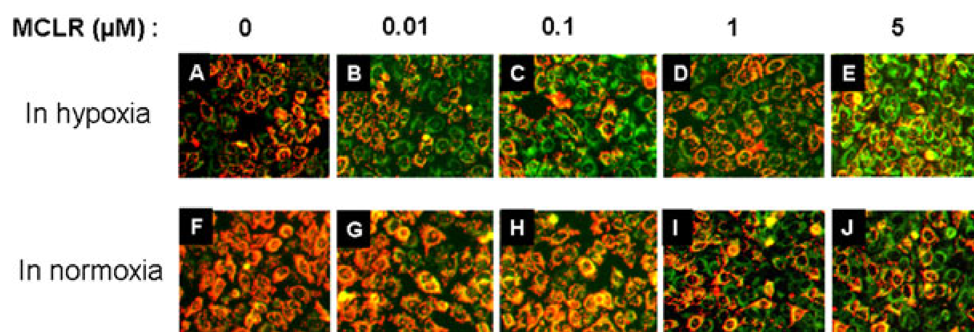

Figure 2. Mitochondrial membrane potential (MMP) changes in HepG2 cells incubated with MC-LR under hypoxic (A–E) and normoxic conditions (F–J). Red fluorescence represents the mitochondrial aggregate form of JC-1 with high MMP and green fluorescence represents the monomeric form of JC-1 with low MMP. (A) The control in hypoxia indicates hypoxia-induced slight MMP loss. (B) 0.01 μM MC-LR, (C) 0.1 μM MC-LR, (D) 1 μM MC-LR and (E) 5 μM MC-LR induced MMP loss in a dose-dependent manner under hypoxic conditions. (F) Control in normoxia represents high MMP in HepG2 cells. (G) 0.01 μM MC-LR, (H) 0.1 μM MC-LR, (I) 1 μM MC-LR and (J) 5 μM MC-LR-induced MMP loss under normoxic conditions.

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MC-LR in hypoxia (Fig. 4C), and up-regulated by MC-LR in a time-dependent manner in normoxia (Fig .4D). The caspase3 mRNA expression in hypoxia (Fig. 4E) was suppressed by 0.01 μM MC-LR at 3 h and all MC-LR concentrations after 6 and 24 h incubation, whereas significantly up-regulated by 0.1 and 1 μM MC-LR at 12 h. The transcription of caspase3 in normoxia (Fig. 4F)

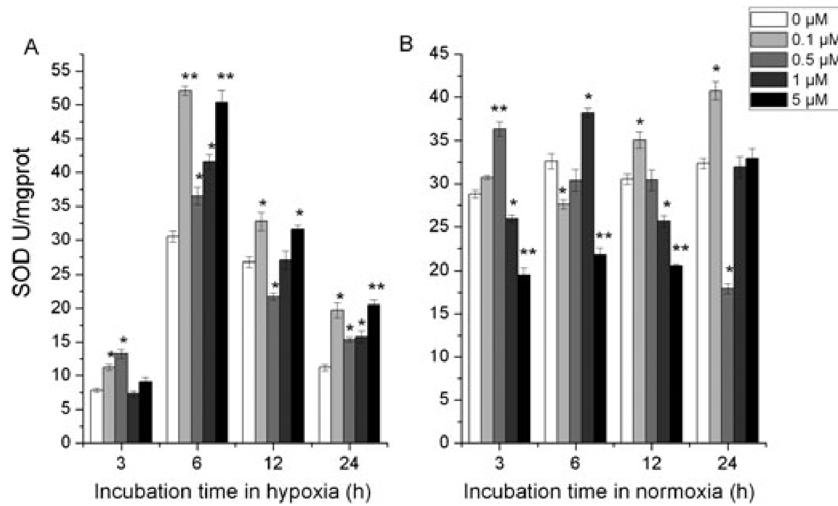

Figure 3. Superoxide dismutase (SOD) activity in HepG2 cells incubated with MC-LR under hypoxic (A) and normoxic (B) conditions. The values are expressed as mean ± standard deviation (SD). *indicates significant differences at $P < 0.05$ and **indicates significant changes at $P < 0.01$ in comparison with control group values.

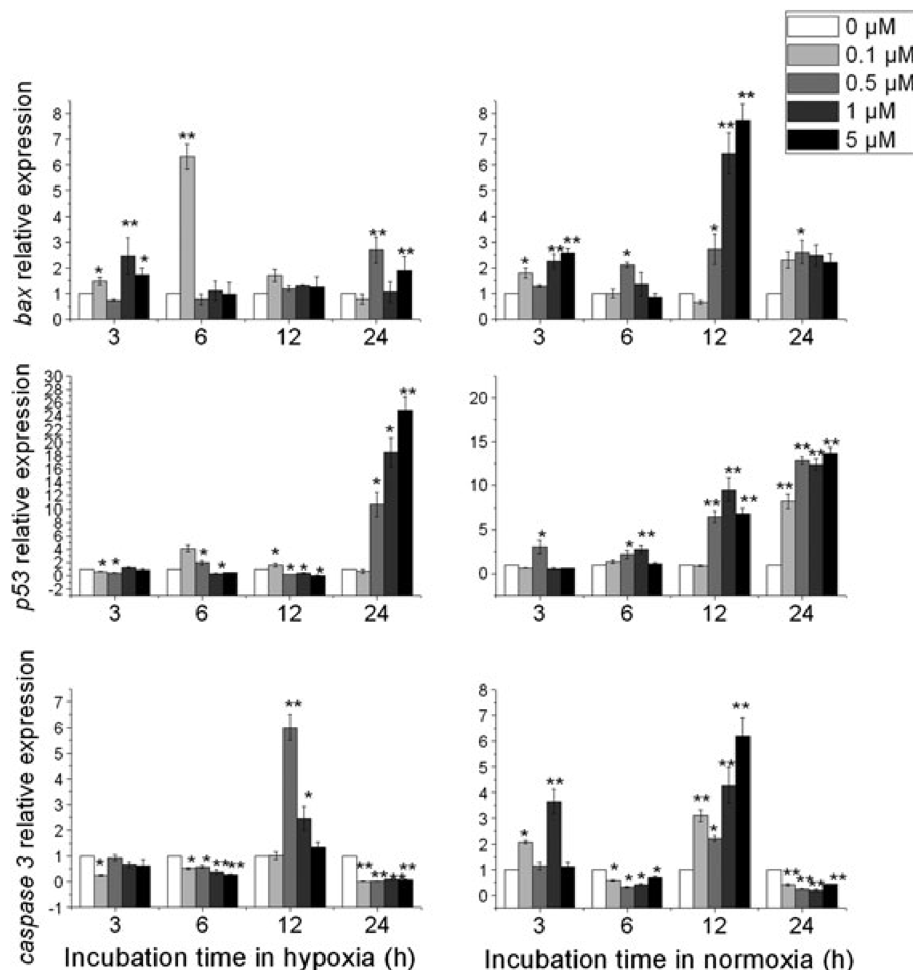

Figure 4. Transcriptional changes of bax (A, B), p53 (C, D) and caspase 3 (E, F) in HepG2 cells incubated with MC-LR under hypoxic (A, C, E) and normoxic (B, D, F) conditions. * indicates significant differences at $P < 0.05$ and **indicates a significant change at $P < 0.01$ in comparison with the control group values.

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was up-regulated by 0.01 and 1 μM MC-LR after 3 h, all MC-LR concentrations at 12 h had reached the maximal value induced by 5 μM MC-LR, but it was down-regulated by all MC-LR concentrations after 6 and 24h exposure.

Western Blot

The changes in inhibitory apoptosis protein c-IAP2 expression are shown in Fig. 5. The expression of c-IAP2 protein was obviously up-regulated at 6 h in hypoxia (Fig. 5A and C), and then fell with further exposure for 12 and 24 h. c-IAP2 protein expression in the hypoxia control group was markedly induced by hypoxia at 6 h but returned to the control level (normoxia control), and then increased at 24 h and higher than that in normoxia control. Under normoxia conditions (Fig. 5B and D), 0.01 μM MC-LR decreased c-IAP2 protein expression at 12 h, although markedly increased at 24h; 0.1 and 1 μM MC-LR significantly up-regulated c-IAP2 protein expression at 6, 12 and 24 h; the highest MC-LR concentration (5 μM) markedly up-regulated c-IAP2 protein expression, but decreased to the control level at 12 h and lower than the control level at 24 h.

Discussion

In the present study, we found that MC-LR showed very different effects on the HepG2 cell line under different oxygen conditions. Under normoxic conditions, MC-LR stimulated proliferation of the HepG2 cell line, which was in agreement with previous studies that MC-LR was a liver tumor promoter and promoted liver cancer cell growth (Gan *et al.*, 2010; Nishiwaki-Matsushima *et al.*, 1992). Under hypoxic conditions, MC-LR induced apoptosis in the HepG2 cell line by inducing prolonged oxidative stress and MMP loss. In addition, we also found that both death and survival signals were activated by MC-LR under hypoxic or normoxic conditions.

Microcystins are liver-specific toxins and evidenced as a tumor promoter in experimental animals (Nishiwaki-Matsushima *et al.*, 1992). In addition, epidemiological studies have suggested that microcystins are one of the risk factors for the high incidence of primary liver cancer in certain areas of China, where people have to drink pond–ditch water contaminated with microcystins (Ueno *et al.*, 1996; Yu, 1995). Recent studies have shown that MC-LR can activate proto-oncogene expression and promote cell proliferation by activating ERK1/2 and Nrf2 signal pathways (Dias *et al.*, 2010; Gan *et al.*, 2010; Li *et al.*, 2009). Under normoxic conditions, we found that MC-LR promoted cell proliferation by activating inhibitory apoptosis protein c-IAP2 expression. c-IAP2 is a member of the family of inhibitors of apoptosis (IAPs), which can directly bind to activated caspase3 and 7 and inhibit their activities (Crook *et al.*, 1993; Duckett *et al.*, 1996). So far, there has been no research carried out on whether MC-LR can affect IAP genes expression. In addition, apoptosis signals such as MMP loss and mRNA transcription of apoptosis-related genes were activated as well. Previous studies have reported that MC-LR could induce MMP loss and apoptosis gene expression (Li *et al.*, 2011; Takumi *et al.* 2010; Zhang *et al.*, 2011). These results suggested that MC-LR induced both death and survival signals in the HepG2 cell line and the survival signal was stronger than death signals induced by MC-LR, indicating that c-IAP2 played an important role in the liver tumor promoter activity of MC-LR.

Oxidative damage is another important mechanism of microcystin toxicity. It is well known that mitochondria is the most

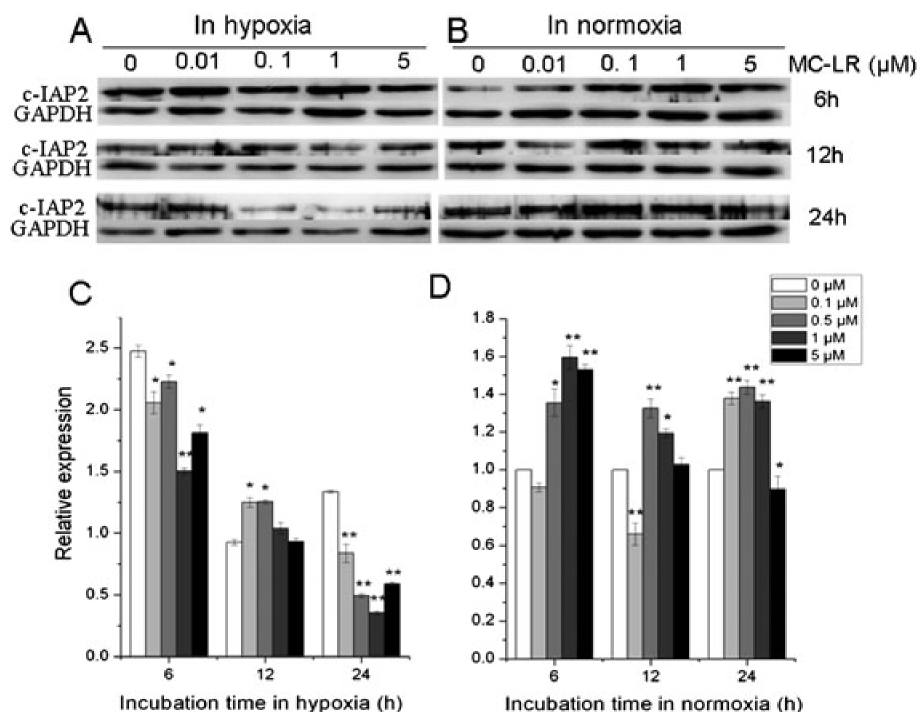

Figure 5. The effect of MC-LR upon the c-IAP2 protein expression in HepG2 cells under hypoxic (A, C) and normoxic (B, D) conditions. (A, B) The representative immunoblot of c-IAP2 protein. (C, D) Quantitation of c-IAP2 protein expression. Mean protein expression in each treated group is shown as a fold increase compared with mean expression in control groups which has been ascribed an arbitrary value of 1. The values are expressed as mean ± standard deviation (SD). * indicates significant differences at $P < 0.05$ and ** indicates a significant change at $P < 0.01$ in comparison with control group values.

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important subcellular site of superoxide production and plays a critical role in O_2 sensors and apoptosis and in mammalian cells (Cadenas and Davies, 2000; Ding *et al*., 1998, 2000; Guzy *et al*., 2005). Previous studies have shown that microcystins induced cell apoptosis by causing intracellular ROS formation and mitochondrial depolarization (Botha *et al*., 2004; Ding *et al*., 2000; Zhang *et al*., 2007). In the present study, we found that MC-LR induced apoptosis of the HepG2 cell line through prolonged oxidative stress and MMP loss under hypoxic conditions, which is very different from the results under normoxic conditions. As a common environmental stress during tumor development, hypoxia can induce apoptosis by increasing hyperpermeability of the mitochondrial inner membrane, generation of ROS, affecting a series of apoptosis-related gene expression (Greijer and van der Wall, 2004; Kietzmann and Gorlach, 2005). Hence, combination of hypoxic stress and MC-LR induced more severe oxidative stress and lower MMP. Besides, anti-apoptosis protein c-IAP2 was also induced by MC-LR after 6 h, then degraded and remained at a lower level than the control. As mentioned before, both death and survival signals were induced by MC-LR stress. However, MC-LR-induced death signals by hypoxic stress were much stronger than those in normoxia.

In conclusion, the present study provided evidence that MC-LR showed a very different effect on the HepG2 cell line in different oxygen concentrations. MC-LR promoted cell growth in normoxia whereas induced cell apoptosis in hypoxia. These results indicated that c-IAP2 contributed to the tumor promoter activity of MC-LR, and severe oxidative was the key factor in causing apoptosis by MC-LR-hypoxia. In addition, we also confirmed that both death and survival signals were activated by MC-LR or hypoxia-MC-LR stresses, and the cell responded to the stronger signals. These results indicated that different oxygen conditions significantly affected MC-LR cytotoxicity.

Conflict of Interest

There is no conflict of interest.

Acknowledgments

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