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Mitochondrial electron transport chain is involved in microcystin-RR induced tobacco BY-2 cells apoptosis

Wenmin Huang[1,2], *Dunhai Li*[1,*], *Yongding Liu*[1]

[1]*Key Laboratory of Algal Biology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China. E-mail: whuangwm@gmail.com*
[2]*Key Laboratory of Aquatic Botany and Watershed Ecology, Wuhan Botanical Garden, the Chinese Academy of Sciences, Wuhan 430074, China*

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ABSTRACT

Microcystin-RR (MC-RR) has been suggested to induce apoptosis in tobacco BY-2 cells through mitochondrial dysfunction including the loss of mitochondrial membrane potential ($\Delta\Psi_m$). To further elucidate the mechanisms involved in MC-RR induced apoptosis in tobacco BY-2 cells, we have investigated the role of mitochondrial electron transport chain (ETC) as a potential source for reactive oxygen species (ROS). Tobacco BY-2 cells after exposure to MC-RR (60 mg/L) displayed apoptotic changes in association with an increased production of ROS and loss of $\Delta\Psi_m$. All of these adverse effects were significantly attenuated by ETC inhibitors including Rotenone (2 μmol/L, complex I inhibitor) and antimycin A (0.01 μmol/L, complex III inhibitor), but not by thenoyltrifluoroacetone (5 μmol/L, complex II inhibitor). These results suggest that mitochondrial ETC plays a key role in mediating MC-RR induced apoptosis in tobacco BY-2 cells through an increased mitochondrial production of ROS.

Introduction

Outbreaks of cyanobacterial (especially *Microcystis aeruginosa*) blooms caused by eutrophication have been a worldwide threat to aquatic ecosystems and human health (Dörr et al., 2010). Microcystins (MCs) are a family of monocyclic nonribosomal peptides produced by cyanobacteria. The generally accepted noxious mechanism of MCs is the inhibition of protein phosphatase (PP) type-1 and 2A. MCs can covalently bind the PP 1 and 2A, thereby influencing regulation of balance between cellular protein phosphorylation and dephosphorylation (Gehringer, 2004). It is thought that MCs affect hepatocellular viability through induction of changes in the cytoskeleton triggered by inhibition of the PPs (Eriksson et al., 1989) and partially through generation of reactive oxygen species (ROS) (Ding et al., 2001). Fladmark et al. (2002) also showed that MCs induced apoptosis correlated with protein phosphorylation events and can be blocked by protein kinase inhibitors.

Recently, the oxidative mechanisms in plants have been well established (Pflugmacher, 2004; Yin et al., 2005; Huang et al., 2008a, 2008b). Oxidative stress is considered as a mediator of apoptosis (Chakraborti et al., 1999). Previous studies have indicated that microcystin-RR (MC-RR) could induce tobacco BY-2 cell apoptosis in a dose- and time-dependent manner mediated by the oxidative stress (Yin et al., 2006). Our studies have further indicated that the mechanism of MC-RR induced apoptosis involves not only the excess generation of ROS and oxidative stress, but also the opening of mitochondrial permeability transition pores (PTP) inducing loss of mitochondrial membrane potential (Huang et al., 2008b).

* *Corresponding author*. E-mail: lidh@ihb.ac.cn (Dunhai Li).

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The mitochondrial electron transport chain (ETC) contains several redox centers that may leak electrons to molecular oxygen, serving as the primary source of ROS in cells (Green and Reed, 1998; Marchi et al., 2012). Under physiological conditions, the ETC has been long suspected to play an important role in apoptosis because mitochondrial ROS was mainly produced from ETC (Green and Reed, 1998). ROS are produced by the ETC at complexes I, II, and III (Hamanaka and Chandel, 2009), which can readily influence mitochondrial function such as the inducement of the mitochondrial PTP opening and collapse of mitochondrial membrane potential (Green and Reed, 1998), without having to cope with long diffusion times from the cytosol. Therefore, the relationship between ETC and MC-RR induced loss of mitochondrial membrane potential as well as apoptosis become major concerns. However, up to date, few studies have assessed the contribution of ETC to MC-RR induced apoptosis in plant cells.

Inhibition of the mitochondrial ETC by inhibitors has been widely used to study the role of ETC in apoptosis (Lee et al., 2006; Murugavel et al., 2007). The level of mitochondrial-derived ROS could be reduced when the mitochondrial ETC was interrupted by ETC inhibitors. Therefore, in this study we have pretreated the tobacco BY-2 cells with ETC inhibitors with the aim to determine both the role of ETC as a potential source of MC-RR induced ROS generation and its role in cell apoptosis.

1. Materials and methods

1.1. Materials

Microcystin-RR was extracted and purified with the improved high performance liquid chromatography with photodiode array detection (HPLC-PDA) (Harada et al., 1988; Lawton et al., 1994). HPLC analysis revealed that the purity of MC-RR was above 95%. Rotenone (Rot), thenoyltrifluoroacetone (TTFA), antimycin A (AA), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), 4′,6′-diamidino-2-phenylindole (DAPI) and propidium iodide (PI) were purchased from Sigma (St. Louis, MO,USA). All cell-culture products from various commercial sources were of analytical or higher grades.

1.2. Cell culture and treatment

The tobacco BY-2 suspension cell line (*Nicotiana tabacum* L. cv. bright yellow 2) were cultured in KCMS liquid medium and maintained as previously specified (Yin et al., 2005). The apoptotic system induced by MC-RR in tobacco BY-2 cells was established according to Huang et al. (2008b). MC-RR was dissolved in deionized water to prepare stock solutions, and then added to the medium to yield a final concentration of 60 mg/L. Three different ETC inhibitors were tested in this study: rotenone (ROT, 2 μmol/L), thenoyltrifluoroacetone (TTFA, 5 μmol/L) and antimycin A (AA, 0.01 μmol/L). Four treatment groups were designed in the study: control group, MC-RR-alone group, inhibitor group and inhibitor-pretreatment + MC-RR group. Cells cultured in KCMS liquid medium were set as control group. Cells treated with 60 mg/L MC-RR were set as MC-RR-alone group. After pretreatment with 2 μmol/L Rot or 5 μmol/L TTFA or 0.01 μmol/L AA for 2 hr, respectively, the cells cultured in KCMS liquid medium were set as inhibitor group. After the same pretreatment, the cells exposed to 60 mg/L MC-RR were set as inhibitor-pretreatment + MC-RR group. Control and treated cells were harvested for morphological assessments and biochemical analysis after 3 and 6 days of culture. All experiments were repeated three times.

1.3. Isolation and preparation of protoplasts

To observe a nuclear morphological change and for flow cytometry analysis, protoplasts were enzymatically isolated from cells as described by Yin et al. (2006) with slight modifications. The cells were subjected to occasional gentle swirling at 30 °C for about 2 hr in an enzyme solution adjusted to pH 5.5 that contained 1% cellulase Onozuka R-10 and 0.1% Pectolyase Y-23. Protoplasts were collected by centrifugation at 800 r/min for 5 min. The number of protoplasts in the suspension was counted with a hemacytometer (Hausser scientific, PA Horsham,USA).

1.4. Morphological observation by a fluorescence microscope

For the evaluation of nuclear morphology, DAPI staining was performed. DAPI was applied to the protoplasts at the final concentration of 10 mg/L in phosphate buffer solution (PBS, pH 7.5) and the protoplasts were incubated for 20 min at room temperature in the dark. Images of the nuclei were obtained using fluorescence microscopy (Nikon ECLIPSE E600,Tokyo, Japan).

1.5. Determination of ROS

Intracellular ROS was detected by using a fluorescent probe, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), according to He and Häder (2002) and Yin et al. (2005) with slight modifications. DCFH-DA (final concentration 5 μmol/L) was added to the cells suspended in 3 mL of 0.1 mol/L PBS (pH 7.8) and the mixture was incubated in an incubator at 25 °C in the dark for 1 hr. The cells were immediately washed three times with PBS (0.1 mol/L, pH 7.8) and finally suspended with 3 mL PBS (0.1 mol/L, pH 7.8). The fluorescence intensity was monitored using a spectrofluorometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA,USA) with excitation wavelength at 485 nm and emission wavelength at 525 nm.

1.6. Determination of mitochondrial membrane potential

Rhodamine 123, a fluorescence probe which selectively enters mitochondria with an intact membrane potential and is retained in the mitochondria, whose mitochondrial fluorescence intensity decreases quantitatively in response to dissipation of the mitochondrial membrane potential, was used to evaluate perturbations in mitochondrial membrane potential (Lemasters et al., 1993). The cells were incubated at 37 °C with 10 μmol/L Rhodamine 123 in an incubator for 30 min with gentle shaking, followed by washing the cells with PBS (0.1 mol/L, pH 7.8). Thereafter, cells were suspended in PBS (0.1 mol/L, pH 7.8) prior to fluorescence measurement with excitation at 485 nm and emission at 530 nm using a spectrofluorometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA,USA).

1.7. Determination of cell apoptosis

Apoptotic cell death was evaluated by double staining with fluorescein isothiocyanate (FITC)-conjugated Annexin V and PI. For this analysis, 1×10^6 cells were stained according to the