JOURNAL OF APPLIED TOXICOLOGY
J. Appl. Toxicol. 2008; **28**: 55–62
Published online 27 April 2007 in Wiley InterScience
(www.interscience.wiley.com) DOI: 10.1002/jat.1251

Mitochondria a key role in microcystin-LR kidney intoxication

R. La-Salete,[1] M. M. Oliveira,[2] C. A. Palmeira,[3] J. Almeida[4] and F. P. Peixoto[1,*]

[1] Chemistry Department, CECAV, University of Trás-os-Montes and Alto Douro, 5001-801 Vila Real, Portugal
[2] Chemistry Center, University of Trás-os-Montes and Alto Douro, 5001-801 Vila Real, Portugal
[3] Mitochondrial Research Group IMAR, Department of Zoology, University of Coimbra, 3000-517 Coimbra, Portugal
[4] Veterinary Department, CECAV, University of Trás-os-Montes and Alto Douro, 5001-801 Vila Real, Portugal

Received 24 January 2007; Revised 1 March 2007; Accepted 5 March 2007

ABSTRACT: Microcystins (MCs) are a group of closely related cyclic heptapeptides produced by a variety of common cyanobacteria. These toxins have been implicated in both human and livestock mortality. Microcystin-LR could affect renal physiology by altering vascular, glomerular and urinary parameters, indicating that MC-LR could act directly on the kidney. The aim of the current work was to examine the effect of MC-LR on mitochondrial oxidative phosphorylation of rat kidney isolated mitochondria.

Furthermore, microcystin-LR decreased both state 3 and carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP)-uncoupled respiration. The transmembrane potential was strongly depressed by MC-LR in a concentration dependent manner, pointing to an uncoupling effect; however, microcystin-LR did not increase the permeability of the inner mitochondria membrane to protons. Therefore, the transmembrane decrease was a consequence of a strong inhibitory effect on redox complexes. The addition of uncoupling concentrations of MC-LR to Ca^{2+}-loaded mitochondria treated with ruthenium red resulted in mitochondrial permeability transition pore (MPTP) opening, as evidenced by mitochondrial swelling in isosmotic sucrose medium. Mitochondrial swelling in the presence of Ca^{2+} was prevented by cyclosporin A and was drastically inhibited by catalase and dithiothreitol, indicating the participation of mitochondrial generated reactive oxygen species in this process. From this study it can be concluded that the bioenergetic lesion promoted by microcystin-LR seems to be sufficient to explain renal injury. Copyright © 2007 John Wiley & Sons, Ltd.

KEY WORDS: microcystin-LR; kidney; mitochondria; mitochondrial permeability transition; bioenergetics

Introduction

Contamination of natural waters by cyanobacterial blooms is a worldwide problem, causing serious water pollution and public health hazards to humans and livestock (Carmichael, 1994; Oudra *et al*., 2001). Members of the cyanobacterial genera *Microcystis*, *Oscillatoria* and *Anabaena* produce cyanotoxins. Microcystins are the most commonly found group of cyanotoxins and more than 70 variants are known (Fastner *et al*., 2002), microcystin-LR being one of the most abundant and toxic variants in blooms.

Microcystin-LR cannot penetrate the cell membrane through simple diffusion, but through the bile acid transport system (Runnegar *et al*., 1991) and by other organic anion transporters localized in hepatocytes and in the blood–brain barrier. These transporters are known to be responsible for the organ-specific toxicity of MC-LR. The disposition of microcystins to hepatotoxic damage probably depends on the high concentration of these transporters in the hepatocyte membrane. The toxicity of microcystins has been attributed to the highly specific inhibition of serine/threonine phosphatases-1 and -2A (Mackintosh *et al*., 1990) and/or to the increased formation of reactive oxygen species (Ding *et al*., 2000). Cytoskeletal changes and apoptosis induced by microcystins were found in experiments on cultured rat hepatocytes and kidney cells (Eriksson *et al*., 1989; Falconer and Yeung, 1992; Wickstrom *et al*., 1995). They have demonstrated that in the intestine microcystin-LR and supernatants of macroph-ages stimulated by this toxin were capable of promoting water and electrolytes secretion (Nobre *et al*., 2004). Acute exposure to MC-LR results in a decrease in the antioxidant enzymes and an increase in lipid peroxidation in rat liver and kidneys, suggesting that oxidative stress has an important role in the pathogenesis of MC-LR-induced toxicity (Ding *et al*., 1998, 2000; Moreno *et al*., 2005). Microcystin-LR induces apoptosis in human derived $CaCo_2$ cell lines and MCF-7 cells via a mechanism similar to that observed for primary rat hepatocytes (Botha *et al*., 2004). Some studies indicate that phospholipase A_2 and arachidonic acid-derived mediators are involved in the renal alterations promoted by microcystin-LR (Nobre *et al*., 2001). Zegura *et al*. (2004) showed that microcystin-LR can induce genotoxicity through ROS formation, which causes the formation of DNA strand breaks and mutagenic oxidative DNA.

* Correspondence to: F. P. Peixoto, Chemistry Department, CECAV, University of Trás-os-Montes and Alto Douro, 5001-801 Vila Real, Portugal.
E-mail: fpeixoto@utad.pt

Studies on the immunohistochemical localization of MC-LR in the liver of poisoned mice, suggest that detection of MC-LR in centrilobular hepatocytes isclosely associated with the onset of hemorrhage and apoptosis and is related to adduct formation (Yoshida *et al*., 1998). Microcystin-treated hepatocytes and other types of cells have typical apoptotic morphology, including chromatin condensation, cell shrinkage and membrane blebbing. A caspase-dependent mechanism should be involved in MC toxicity, since caspase inhibitors could retard the execution (McDermott *et al*., 1998; Fladmark *et al*., 1999). However, the exact mechanisms underlying the suggested apoptosis-induced potential are still unknown. Involvement of mitochondria, including an increase of reactive oxygen species followed by a loss of mitochondrial membrane potential was observed (Ding *et al*., 2000). Moreover, mitochondria have been found to be one of the major targets in the MC-LR induced apoptotic process, and the onset of mitochondrial permeability transition (MPT) precedes the characteristic signs of apoptosis (Ding *et al*., 2000). Ding *et al*. (2001) showed that MC-LR caused an early surge of mitochondrial Ca^{2+} prior to the onset of MPT and cell death in MC-LR treated rat hepatocytes. Immunochemical and proteomic studies have enabled the identification of type-1 catalytic subunits, type-2A protein phosphatases and ATP-synthase beta subunits as targets for microcystin-LR (Mikhailov *et al*., 2003).

The detoxification of MC-LR in the liver occurs through conjugation with glutathione (Kondo *et al*., 1996) via glutathione S-transferase action (Pflugmacher *et al*., 1998; Takenaka, 2001).

Recent studies have demonstrated that microcystin-LR-stimulated macrophages cause significant increases in renal vascular resistance, in glomerular filtration rate and in urinary flow. Furthermore, they release mediators capable of promoting nephotoxicity in isolated perfused rat kidney (Nobre *et al*., 1999), involving phospholipase A_2 and a cyclooxygenase in this process (Nobre *et al*., 2003). Many authors have already shown that MC-LR promotes renal alterations and affects renal physiology (Wickstrom *et al*., 1995; Nobre *et al*., 1998; Beasley *et al*., 2000; Fischer and Dietrich, 2000; Milutinovic *et al*., 2002). Since renal mitochondrial bioenergetics plays a crucial role in cellular metabolism, the main objective of this work was to evaluate the effects of microcystin-LR on isolated kidney mitochondria.

Materials and Methods

Isolation of Rat Kidney Mitochondria

Mitochondria were extracted from a homogenate of rat kidney by differential centrifugation according to Cain and Skilleter (1987).

Wistar rats (male, weighing 300–400 g) were killed by decapitation and the kidneys excised. The entire organs were homogenized (after first removing the capsule) in an ice-cold isolation medium containing 0.25 M sucrose, 20 mM HEPES, 1 mM ethylene-diaminetetraacetic (EDTA), 0.2% (w/v) defatted bovine serum albumin, pH 7.4, and homogenized (10 ml g^{-1}) using a Potter-Elvejhem homogenizer. Mitochondria isolation was performed at 4 °C without delay using differential centrifugation. Nuclei and unbroken cells were pelleted by centrifugation at 700 *g* for 10 min, and the mitochondria were separated from the supernatant by centrifugation at 10 000 *g* for 10 min. The mitochondrial pellet was washed twice and resuspended in a medium containing sucrose 0.25 M, 20 mM HEPES, pH 7.4 and suspended at 15–30 mg of protein ml^{-1}. The protein concentration of mitochondrial suspension was determined by the biuret method (Gornall *et al*., 1949), in the presence of 0.2% DOC, using BSA as a standard protein.

Mitochondrial Respiratory Activity

Oxygen consumption of isolated mitochondria was measured polarographically using a Clark-type oxygen electrode (Estabrook, 1967) connected to a suitable recorder in a closed water-jacketed 1.0 ml chamber with magnetic stirring, at 25 °C. Respiration rates were calculated assuming an oxygen concentration of 450 nAt O ml^{-1} in the experimental medium at 25 °C. The standard respiratory medium consisted of 130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$, 5 mM KH_2PO_4 and 5 mM Hepes, pH 7.2. Microcystin-LR was added in aliquots (a few microliters) to 1 ml of the standard respiratory medium (25 °C) supplemented with mitochondria (0.5 mg protein) and allowed to incubate for 10 min before the addition of respiratory substrate, i.e. before the beginning of the respiratory activity. In all the assays a negative control was included to evaluate the effect of the ethanol, nevertheless the volumes used did not induce any significant toxicity. State 3 was elicited by adding adenosine 5′-diphosphate (ADP 1 mM) and uncoupled respiration, by adding 1 μM carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP).

Membrane Potential

A tetraphenylphosphonium (TPP^+)-sensitive electrode, prepared according to Kamo *et al*. (1979), was used to estimate the potential across the mitochondrial membrane. TPP^+ uptake was measured from the decreased TPP^+ concentration in the medium sensed by the electrode. The potential difference between the selective electrode and the reference (calomel electrode) was measured with an electrometer and the data were collected in a Hansatech

DOI: 10.1002/jat

acquisition data system. The voltage response of the TPP^+ electrode to log (TPP^+) was linear with a slope of 59 ± 1, at 25 °C, equating well with the Nernst equation.

Mitochondria (0.5 mg protein) were re-suspended in 1 ml of the standard respiratory medium (the same medium as described for the oxygen consumption experiments), at 25 °C, with constant stirring. TPP^+ was added to a final concentration of 3 μM, i.e. an amount low enough to avoid any detectable toxic effects on mitochondria with the respiratory substrates. The TPP^+ uptake was initiated by the addition of substrate. The $\Delta\psi$ was estimated as indicated by Kamo *et al*. (1979) from the equation

$$\Delta\psi\ (\text{mV}) = 59\ \log(v/V) - 59\ \log(10^{\Delta E/59} - 1)$$

where v, V and ΔE are the volumes of mitochondrial and incubation medium and the deflection of the electrode potential from the baseline, respectively. A matrix volume of 1.1 μl mg^{-1} mitochondrial protein was considered. The above equation was derived assuming that the TPP^+ distribution between the mitochondria and the medium followed the Nernst equation and that the law of mass conservation was applicable. No correction was made for the passive binding of TPP^+ to the mitochondrial membranes, since the purpose of the experiments was to show relative changes in potentials rather than absolute values. Calibration runs in the presence of MC-LR excluded any direct interference in the electrode signal.

Synthesis of Decylubiquinol

Decylubiquinol was synthesized according to the method of Gudz *et al*. (1997; 2031) and all procedures were carried out in subdued light. Decylubiquinone (10 μmol) was dissolved in 2 ml ethanol + water (1 + 1 v/v; pH 2) and reduced to the corresponding alcohol by the addition of $NaBH_4$. The decylubiquinol was twice extracted from the aqueous ethanol using 1 ml of diethylether + isooctane (2 + 1; v/v). The organic phases were combined, then washed with 2 ml of 2 M NaCl and evaporated to dryness at room temperature under a stream of nitrogen. The product was dissolved in ethanol (990 μl), acidified by the addition of 10 μl of 0.1 M HCl and transferred to a storage vial. After flushing the airspace of the vial with nitrogen, the vial was securely capped and the decylubiquinol solution kept at −20 °C in darkness.

Enzymatic Activities

Complex II activity was measured as the rate of reduction of ubiquinone to ubiquinol by succinate, and quantified by the secondary reduction of 2,6-dichlorophenolindophenol (DCIP) as the quinol formed. The reaction mixture contained 50 mM potassium phosphate buffer (1 ml; pH 7.6), 20 mM succinate, 1.0 mM EDTA, 0.05 mM DCIP and 3 mM sodium azide. Decylubiquinone (50 μM) was added followed by mitochondria (65 μg) to start the reaction. The decrease in absorbance as DCIP became reduced was measured at 600 nm. Complex III activity was assayed as an increase in absorbance at 550 nm as cytochrome c was reduced by complex III with decylubiquinol present as the electron donor. The reaction mixture consisted of 50 mM potassium phosphate buffer (1 ml; pH 7.6) containing 0.1% BSA, 0.1 mM EDTA, 60 μM cytochrome c, 3 mM sodium azide and decylubiquinol (150 μM). Mitochondria (10 μg protein) were then added to initiate the reaction. Complex IV activity was measured with reduced cytochrome c as substrate. The reaction mixture consisted of 50 mM potassium phosphate buffer (1 ml; pH 7.6) containing sucrose (0.25 mM) and 25 μM reduced cytochrome c. Mitochondria (10 μg protein) were introduced to start the reaction and the oxidation of cytochrome c was monitored at 550 nm. Cytochrome c was reduced with sodium dithionite prior to assay.

ATP-synthase activity was determined by monitoring the pH increase associated with ATP synthesis (Madeira *et al*., 1974). The reaction was carried out in 2 ml of the reaction medium containing 130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$ and 2 mM KH_2PO_4 (pH 7.2), supplemented with 5 mM succinate and 1 mg of mitochondrial protein. The reaction was initiated by the addition of 200 μM ADP to the mitochondrial suspension. The pH change was evaluated with a Crison pH meter connected to a Hansatech acquisition data system. The addition of oligomycin (2 μg mg^{-1} protein) completely halted H^+ consumption. H^+ consumption was calculated after an elapsed time of 2 min from the start of the reaction.

ATPase activity was determined by monitoring the pH change associated with ATP hydrolysis (Madeira *et al*., 1974). The reaction was carried out in 2 ml of a medium containing 130 mM sucrose, 50 mM KCl, 5 mM $MgCl_2$ and 0.5 mM Hepes (pH 7.2), supplemented with 2 μM rotenone and mitochondria (1 mg protein of disrupted mitochondria). The reaction was initiated by the addition of 2 mM Mg-ATP and was completely inhibited by the addition of oligomycin (2 μg mg^{-1} mitochondrial protein), meaning that the activity measured was due to a mitochondrial F_0-F_1 ATPase which is a Mg^{2+} ATPase. Proton production was again calculated 2 min after starting the reaction.

Mitochondrial Swelling

Mitochondrial osmotic swelling was monitored by detecting turbidity, at 520 nm, on a Varian 50 spectrophotometer, in a thermostatic chamber with magnetic stirring at 25 °C. To detect the mitochondrial protonophoric effect induced by MC-LR, assays were carried out at 25 °C in 2.5 ml of NH_4NO_3 medium (135 mM NH_4NO_3, 0.1 mM

DOI: 10.1002/jat

EDTA, 5 mM HEPES, pH 7.2), supplemented with 2 μM rotenone and 0.5 mg mitochondria. MC-LR was added in DMSO solution and incubated for 5 min.

Chemicals

All reagents were of analytical grade commercially available.

Statistics

The results are presented as a percentage of the controls ± SEM from at least three independent experiments. Statistical analyses were performed using two-tailed unpaired *t*-tests. A value of $P < 0.05$ was considered statistically significant. Some figures are records of individual experiments representative of three or more replicates.

Results

Effects of Microcystin-LR on Kidney Mitochondrial Respiration

The concentration-dependent effects of MC-LR on respiratory rates, characteristics of state 4 (after complete phosphorylation of 25 nmol of ADP), state 3 respiration (ADP-stimulated) and uncoupled respiration (FCCP-stimulated) are illustrated in Fig. 1. Control values for succinate supported-respirations are expressed in nmol O min^{-1} mg^{-1} protein. State 4, 56.6 (±10.2); state, 3 169.6 (±25.4); uncoupled respiration, 209 (±12.1). The respiratory control (RCR), and coupling oxidative phosphorylation (ADP/O ratio) were 3.0 (±0.1) and 1.8 (±0.2), respectively. State 4 respiration of rat kidney mitochondria was significantly stimulated (40% of the control) by the MC-LR at concentrations above 15 nmol mg^{-1} (mitochondrial protein), but at higher concentrations a significant inhibition was observed. When the mitochondria were incubated in a reaction medium containing 200 nmol ADP or FCCP at a concentration that promotes maximal uncoupling (Heytler and Prichard, 1962), the treatment with microcystin-LR (50 nmol MC-LR mg^{-1} mitochondrial protein) produced a strong inhibition of the succinate-sustained oxygen uptake of about 46% and 33% of the control, respectively, in state 3 and uncoupled respiration.

Effects of Microcystin-LR on Kidney Mitochondrial Membrane Potential

Mitochondria developed a normal transmembrane potential ($\Delta\psi$) of about 200 mV (negative inside) upon the addition of a respiratory substrate. The presence of MC-LR in the reaction medium depressed the $\Delta\psi$ promoted by succinate when compared with the control (Fig. 2), this effect being dependent on the concentration of MC-LR. Microcystin-LR at the maximum concentration used in this assay (30 nmol mg^{-1} mitochondrial protein), decreased the initial $\Delta\psi$ by about 20% of the control. Furthermore, the $\Delta\psi$ decreased strongly with time, collapsing after 10 min of succinate-energization (for the highest concentration of MC-LR used).

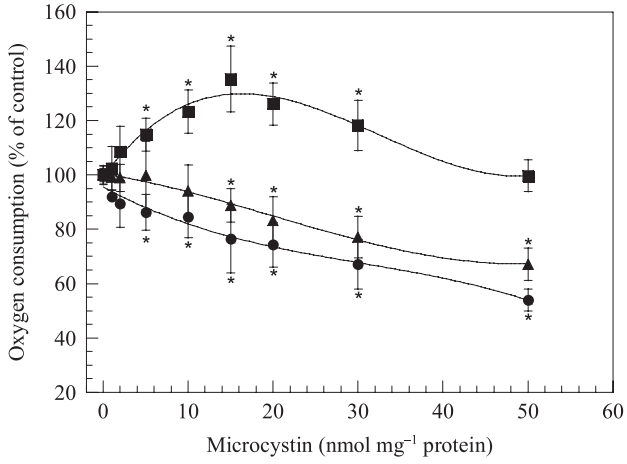

Figure 1. Effect of MC-LR on respiratory rates of mitochondria. Mitochondria (0.5 mg) were incubated, for 10 min, in 1 ml of the respiratory standard medium, supplemented with rotenone (2 μM), in the presence of different MC-LR concentrations. (■) State 4 respiration was initiated by the addition of 15 mM succinate, (▲) State 3 respiration energized by 5 mM succinate was initiated by the addition of 1.5 mM ADP, (●) FCCP-uncoupled respiration in the presence of 15 mM succinate was initiated by the addition of 1 μM FCCP. ADP or FCCP were added 2 min after the initiation of state 4 respiration. Values are the mean ± SD of six independent experiments performed in duplicates. * Values statistically different from control ($P < 0.05$)

Enzymatic Activities

In order to determine whether the effect of microcystin-LR on kidney mitochondria bioenergetics is by a direct action on the respiratory complexes or by an uncoupler effect, the effect of this compound on respiratory complexes (II, III and IV) and in the mitochondria swelling was evaluated.

The inhibitory effect of MC-LR, on the succinate-supported respiration at state 3, uncoupled respiration and also at state 4 respiration for MC-LR concentrations above 15 nmol mg^{-1} protein (Fig. 1), suggests that

DOI: 10.1002/jat

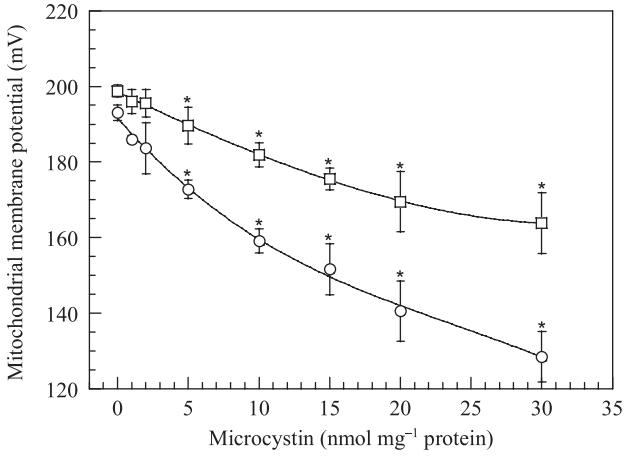

Figure 2. Effect of MC-LR on transmembrane potential ($\Delta\psi$) supported by succinate. Mitochondria (0.5 mg) were added to the standard respiratory medium supplemented with 3 μM TPP^+. Maximum potential reached due succinate oxidation after 10 min incubation with MC-LR (□) and potential reached after 10 min succinate-respiration (○). Values are the mean ± SD of six independent experiments performed in duplicates. * Values statistically different from control ($P < 0.05$)

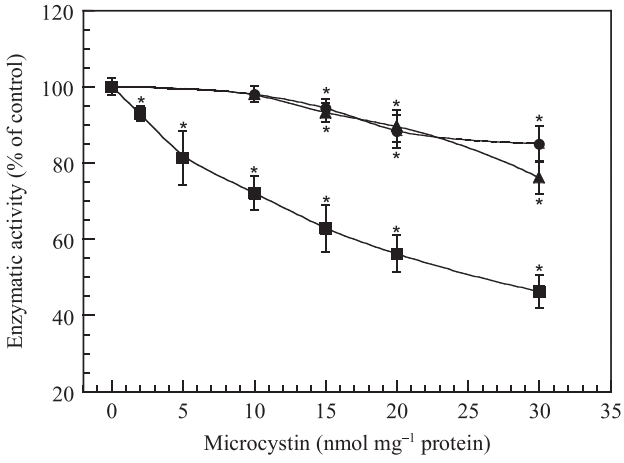

Figure 3. Effect of microcystin-LR on the respiratory complexes succinate dehydrogenase (▲), succinate cytochrome *c* reductase (■) and cytochrome c oxidase (●). The activities were determined as described in Materials and Methods. Values are the mean ± SD of four independent experiments performed in duplicates. * Values statistically different from control ($P < 0.05$)

it may be due to an inhibition on the respiratory complexes and a quite probable inhibition on the phosphorylative system (F0F1-ATPase/ATP synthase). Indeed, the experiments illustrated in Fig. 4 showed that MC-LR (30 nmol mg^{-1} mitochondrial protein) induced an inhibition of ATPase and ATP synthase activities of about 45% and 80%, respectively. Furthermore, all the tested concentrations induced statistically significant alterations (Fig. 4).

Succinate dehydrogenase activity was significantly inhibited by microcystin treatment at concentrations higher than 15 nmol mg^{-1} protein. At the maximum concentration (30 nmol mg^{-1} protein) MC-LR inhibited succinate dehydrogenase by 54% compared with the control (Fig. 3). Moreover, succinate cytochrome oxidase was similarly inhibited by low MC-LR concentrations, but at the highest concentration used (30 nmol mg^{-1} protein) the inhibition was lower by about 15% when compared with the observed inhibition for succinate dehydrogenase.

From the results obtained for ATPase and ATP synthase (Fig. 4) it can be seen that both enzymatic activities were inhibited by MC-LR in a concentration dependent manner. ATP synthase was more inhibited than ATPase. At a concentration of 30 nmol mg^{-1} protein, ATPase and ATP synthase activities were inhibited by about 46% and 81% compared with the control. The differences observed for these two enzymatic activities were statistically significant for MC-LR concentrations equal to or higher than 5 nmol mg^{-1} protein.

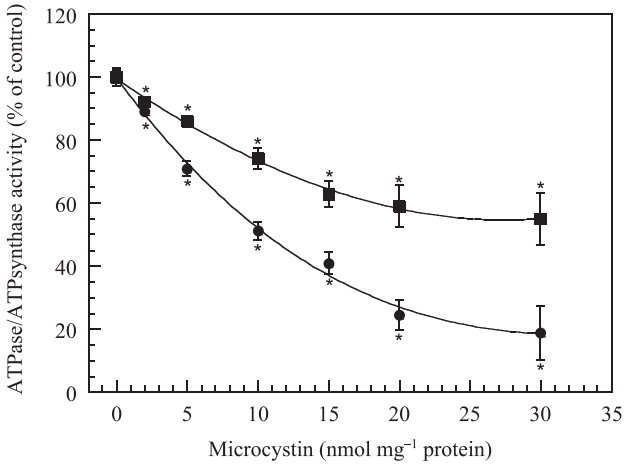

Figure 4. Effect of MC-LR at different concentrations on ATPase/ATPsynthase activity. Experimental conditions are described in Materials and Methods. Values are the mean ± SD of four independent experiments performed in duplicates. * Values statistically different from control ($P < 0.05$)

Effects of Microcystin-LR on Mitochondrial Permeability Transition Pore Induction and on Proton Leak

The mitochondria permeability transition pore participates in the regulation of matrix Ca^{2+}, pH, mitochondrial membrane potential and volume. Microcystin increased the susceptibility to induction of permeability transition (Fig. 5) in calcium-loaded mitochondria. The mitochondria permeability transition pore was rapidly opened by MC-LR (90 nmol mg^{-1} protein) added to mitochondria

DOI: 10.1002/jat

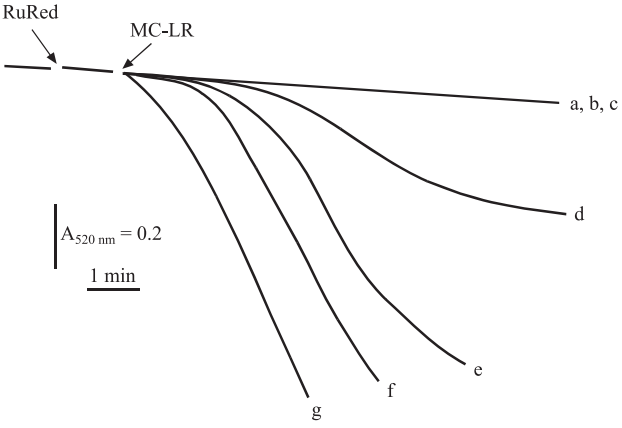

Figure 5. Mitochondrial permeability transition induced by microcystin-LR on Ca^{2+}-loaded (150 nmol mg^{-1} protein) mitochondria treated with ruthenium red. Mitochondria (1 mg) were added to 2 ml of reaction medium (250 mM sucrose, 10 mM HEPES-Na^+ buffer pH 7.2, 1 mM KH_2PO_4, supplemented with 4 μM rotenone, 0.5 μg oligomycin ml^{-1} and energized with 5 mM succinate, at 25 °C) in the presence of: (line a) 1 μM CsA (line c) 2 mM DTT or (lines a–f) Ca^{2+} (150 nmol). Ruthenium red (1 μM) was added where indicated, followed by the addition of MC-LR (nmol mg^{-1} protein) in the following concentrations: (lines a, and c) 50, (line b) 0, (line d) 15, (line e) 30, (line f) 60 and (line g) 90. The traces represent typical recordings

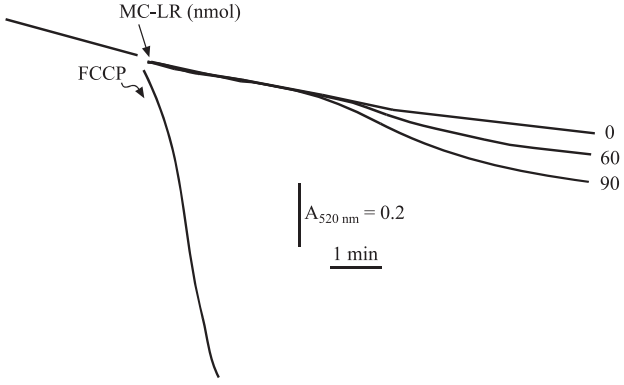

Figure 6. Effect of MC-LR on mitochondrial swelling in isoosmotic NH_4NO_3 medium. Mitochondria (1 mg) were incubated for 5 min with MC-LR at the indicated concentrations in 2 ml of NH_4NO_3 medium (135 mM NH_4NO_3, 0.1 mM EDTA, 5 mM HEPES, pH 7.2) supplemented with 2 μM rotenone. The reactions were carried out at 25 °C. The traces represent typical recordings

preloaded with calcium and treated with ruthenium red to prevent the early mitochondrial calcium surge. However, pretreatment with cyclosporin A (1 μM) or with DTT (2 mM), completely inhibited the effect of pretreatment with 60 nmol mg^{-1} protein MC-LR (Fig. 5). Proton leaks induced by MC-LR through the mitochondrial inner membrane were investigated by swelling in isoosmotic NH_4NO_3 (Fig. 6). At the tested conditions no mitochondrial protonophoric effect was observed after treatment with MC-LR.

Discussion

The present work addresses the very first report of the effect of microcystin-LR on mitochondrial oxidative phosphorylation of rat kidney isolated mitochondria. Several studies have reported that microcystins induce severe hepatic hemorrhage leading to shock (Runnegar and Falconer, 1982) but also can affect rat kidney, indicating microcystin to be a potential nephrotoxic (Eriksson *et al*., 1988, 1989; Hooser *et al*., 1989; Nobre *et al*., 1999; Milutinovic *et al*., 2003). Some conspicuous effects of microcystin-LR were also been reported on carp kidney (Rabergh *et al*., 1991). Many other studies show the nephrotoxicity promoted by microcystin-LR (Kotak *et al*., 1996; Nobre *et al*., 1999; Milutinovic *et al*., 2002, 2003).

Human intoxications were described for populations exposed through drinking water or recreational activities (Falconer, 1999) and for many patients submitted to renal dialysis treatment (Pouria *et al*., 1998; Azevedo *et al*., 2002; Soares *et al*., 2006).

Mitochondria are known to be the vulnerable target of microcystin (Ding and Ong, 2003). Microcystin has been shown to rapidly decrease the MMP in hepatocytes before LDH leakage, suggesting that membrane potential depolarization is an early event of microcystin-induced hepatotoxicity (Ding *et al*., 2001). It was also demonstrated that microcystin could bind to ATP synthase, an important component in the mitochondria (Mikhailov *et al*., 2003). Human liver mitochondrial aldehyde dehydrogenase is also a potential target for microcystin-LR (Chen *et al*., 2006), pointing to the relevance of mitochondria in microcystin toxicity.

The present study demonstrated that MC-LR interacted with mitochondrial oxidative phosphorylation. Mitochondrial basic functions were monitored through respiration and mitochondrial membrane potential (MMP). The generation of MMP is due to the asymmetric distribution of protons and other ions on both sides of the inner mitochondrial membrane, giving rise to a chemical and electrical gradient. The MMP alterations in rat kidney mitochondria were examined using a TPP^+-electrode. Microcystin has been shown to decrease the maximum MMP achieved by succinate oxidation, but also a strong inhibitory decrease of MMP which was time and concentration dependent (Fig. 2). The absence of a proton leak was confirmed by monitoring the mitochondrial swelling in isoosmotic NH_4NO_3 (Fig. 6). In fact, the permeability of mitochondria to protons was slightly increased by MC-LR (90 nmol mg^{-1} protein), a small effect when compared

DOI: 10.1002/jat

with that of FCCP (Fig. 6). Therefore the decrease of the transmembrane potential induced by MC-LR can simply be the result of an inhibition of the respiratory complexes or of the translocation of redox equivalents from succinate to the inside of the mitochondria. Furthermore, the inhibitory effect observed in the succinate-supported uncoupled respiration (FCCP-stimulated) was already a good indicator of an inhibitory effect at the respiratory complexes level (II, III or IV).

In an attempt to elucidate the exact action of MC-LR on the respiratory redox chain and the phosphorylative system, studies were made as previously explained (Fig. 3). From these results it can be concluded that the effect was mostly localized on succinate cytochrome c reductase, since mitochondrial incubation with 30 nmol MC-LR mg^{-1} protein, decreased the activity by about 55% compared with the control. Whereas at this concentration succinate dehydrogenase and succinate cytochrome c oxidase activities were only inhibited by 25% and 15% of the control, respectively. Furthermore, the phosphorylative system was also strongly affected by MC-LR treatment (Fig. 4), since MC-LR (30 nmol mg^{-1} protein) decreased the ATP synthase and ATPase activities by about 80% and 40% of the control, respectively.

It has long been known that mitochondrial Ca^{2+} overload leads to the opening of the mitochondrial permeability transition pore (MPT) (Nicholls, 1982; Gunter and Pfeiffer, 1990; Zoratti and Szabò, 1995). MPT opening can be enhanced by various Ca^{2+} releasing agents, such as inorganic phosphate (Pi), arsenate, fatty acids and thiol oxidants (Nicholls, 1982; Gunter and Pfeiffer, 1990; Gunter *et al*., 1994; Zoratti and Szabò, 1995). MPT, also called the mitochondrial megachannel, represents an abrupt increase of permeability of the mitochondrial inner membrane to solutes of molecular mass less than 1500 Da (Lemasters *et al*., 1998). Ding *et al*. (2001) have already demonstrated the effect of MC-LR on inducing MPT in rat hepatocytes, however, the effect on isolated mitochondria has never been reported. The data clearly showed that MC-LR induced MPT in isolated kidney mitochondria (Fig. 5). Furthermore, this MPT can be inhibited by cyclosporin A and DTT, which unequivocally proves that the observed swelling induced by MC-LR was due to MPT pore.

Miura *et al*. (1989) have previously demonstrated that liver mitochondria isolated from fasted rats treated with MC-LR showed alterations in appearance which was correlated with a loss of coupled electron transport. Liver mitochondria from toxin treated, fasted rats exhibited complete inhibition of state 3 respiration, while those from toxin-treated, fed rats had ADP/O ratios and respiratory control indices comparable to the control values (Miura *et al*., 1991).

Contrary to the *in vitro* results obtained in rat liver mitochondria by Miura *et al*. (1989), where no effects were observed for the tested concentrations, the present study showed significant effects on the *in vitro* studies with rat kidney isolated mitochondria treated with MC-LR.

In conclusion, the data clearly indicate that the toxicological effect of MC-LR is due to inhibition of the mitochondrial phosphorylative system and respiratory chain, which ultimately lead to disruption of cellular bioenergetics and metabolism. This energetic disruption at the mitochondrial level, is mainly responsible for the renal physiological and metabolic alterations reported by several authors (Nobre *et al*., 2001, 2003; Milutinovic *et al*., 2003; Moreno *et al*., 2005).

References

Azevedo SMFO, Carmichael WW, Jochimsen EM, Rinehart KL, Lau S, Shaw GR, Eaglesham GK. 2002. Human intoxication by microcystins during renal dialysis treatment in Caruaru-Brazil. *Toxicology* **181**: 441–446.

Beasley VR, Lovell RA, Holmes KR, Walcott HE, Schaeffer DJ, Hoffmann WE, Carmichael WW. 2000. Microcystin-LR decreases hepatic and renal perfusion, and causes circulatory shock, severe hypoglycemia, and terminal hyperkalemia in intravascularly dosed swine. *J. Toxicol. Environ. Health Part A* **61**: 281–303.

Botha N, Gehringer MM, Downing TG, van de Venter M, Shephard EG. 2004. The role of microcystin-LR in the induction of apoptosis and oxidative stress in CaCo2 cells. *Toxicon* **43**: 85–92.

Cain K, Skilleter DN. 1987. *Preparation and Use of Mitochondria in Toxicological Research*. Oxford: Washington DC.

Carmichael WW. 1994. Toxins of cyanobacteria. *Sci. Am.* **270**: 78–86.

Chen T, Cui J, Liang Y, Xin XB, Young DO, Chen C, Shen PP. 2006. Identification of human liver mitochondrial aldehyde dehydrogenase as a potential target for microcystin-LR. *Toxicology* **220**: 71–80.

Ding WX, Ong CN. 2003. Role of oxidative stress and mitochondrial changes in cyanobacteria-induced apoptosis and hepatotoxicity. *FEMS Microbiol. Lett.* **220**: 1–7.

Ding WX, Shen HM, Ong CN. 2000. Critical role of reactive oxygen species and mitochondrial permeability transition in microcystin-induced rapid apoptosis in rat hepatocytes. *Hepatology* **32**: 547–555.

Ding WX, Shen HM, Ong CN. 2001. Pivotal role of mitochondrial Ca^{2+} in microcystin-induced mitochondrial permeability transition in rat hepatocytes. *Biochem. Biophys. Res. Commun.* **285**: 1155–1161.

Ding WX, Shen HM, Zhu HG, Ong CN. 1998. Studies on oxidative damage induced by cyanobacteria extract in primary cultured rat hepatocytes. *Environ. Res.* **78**: 12–18.

Eriksson JE, Meriluoto JAO, Kujari HP, Skulberg OM. 1988. A comparison of toxins isolated from the *Cyanobacteria oscillatoria Agardhii* and *Microcystis aeruginosa*. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* **89**: 207–210.

Eriksson JE, Paatero GIL, Meriluoto JAO, Codd GA, Kass GEN, Nicotera P, Orrenius S. 1989. Rapid microfilament reorganization induced in isolated rat hepatocytes by microcystin-LR, a cyclic peptide toxin. *Exp. Cell Res.* **185**: 86–100.

Estabrook RW. 1967. In *Membranes of Mitochondria and Chloroplasts*, Racker E (ed.), Van Nostrand: New York; 172–212.

Falconer IR. 1999. An overview of problems caused by toxic blue-green algae (cyanobacteria) in drinking and recreational water. *Environ. Toxicol.* **14**: 5–12.

Falconer IR, Yeung DSK. 1992. Cytoskeletal changes in hepatocytes induced by microcystis toxins and their relation to hyperphosphorylation of cell-proteins. *Chem. Biol. Interact.* **81**: 181–196.

Fastner J, Codd GA, Metcalf JS, Woitke P, Wiedner C, Utkilen H. 2002. An international intercomparison exercise for the determination of purified microcystin-LR and microcystins in cyanobacterial field material. *Anal. Bioanal. Chem.* **374**: 437–444.

Fischer WJ, Dietrich DR. 2000. Pathological and biochemical characterization of microcystin-induced hepatopancreas and kidney damage in carp (*Cyprinus carpio*). *Toxicol. Appl. Pharmacol.* **164**: 73–81.

Fladmark KE, Brustugun OT, Hovland R, Boe R, Gjertsen BT, Zhivotovsky B, Doskeland SO. 1999. Ultrarapid caspase-3 dependent apoptosis induction by serine/threonine phosphatase inhibitors. *Cell Death Differ*. **6**: 1099–1108.

Gornall AG, Bardawill CJ, David MM. 1949. Determination of serum proteins by means of the biuret. *J. Biol. Chem.* **177**: 751–766.

Gudz TI, Tserng KY, Hoppel CL. 1997. Direct inhibition of mitochondrial respiratory chain complex III by cellpermeable ceramide. *J. Biol. Chem.* **272**: 24154–24158.

Gunter TE, Gunter C, KK, Sheu SS, Gavin CE. 1994. Mitochondrial Ca^{2+} transport: physiological and pathological relevance. *Am. J. Physiol.* **267**: C313–C339.

Gunter TE, Pfeiffer DR. 1990. Mechanisms by which mitochondria transport calcium. *Am. J. Physiol.* **258**: C755–C786.

Heytler PG, Prichard WW. 1962. A new class of uncoupling agents – carbonylcyanide phenylhydrazones (CCP). *Biochem. Biophys. Res. Commun.* **7**: 272–275.

Hooser SB, Beasley VR, Lovell RA, Carmichael WW, Haschek WM. 1989. Toxicity of microcystin LR, a cyclic heptapeptide hepatotoxin from *Microcystis aeruginosa*, to rats and mice. *Vet. Pathol.* **26**: 246–252.

Kamo N, Muratsugu M, Hongoh R, Kobatake V. 1979. Membrane potential of mitochondria measured with an electrode sensitive to tetraphenylphosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. *J. Membr. Biol.* **49**: 105–121.

Kondo F, Matsumoto H, Yamada S, Ishikawa N, Ito E, Nagata S, Ueno Y, Suzuki M, Harada K. 1996. Detection and identification of metabolites of microcystins formed *in vivo* in mouse and rat livers. *Chem. Res. Toxicol.* **9**: 1355–1359.

Kotak BG, Semalulu S, Fritz DL, Prepas EE, Hrudey SE, Coppock RW. 1996. Hepatic and renal pathology of intraperitoneally administered microcystin-LR in rainbow trout (*Oncorhynchus mykiss*). *Toxicon* **34**: 517–525.

Lemasters JJ, Nieminen AL, Qian T, Trost LC, Elmore SP, Nishimura Y, Crowe RA, Cascio WE, Bradham CA, Brenner DA, Herman B. 1998. The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. *Biochim. Biophys. Acta* **1366**: 177–196.

Mackintosh C, Beattie KA, Klumpp S, Cohen P, Codd GA. 1990. Cyanobacterial Microcystin-LR is a potent and specific inhibitor of protein phosphatase-1 and phosphatase-2a from both mammals and higher-plants. *FEBS Lett.* **264**: 187–192.

Madeira VMC, Antunes-Madeira MC, Carvalho AP. 1974. Activation energies of the ATPase activity of sarcoplasmic reticulum. *Biochem. Biophys. Res. Commun.* **58**: 897–904.

McDermott CM, Nho CW, Howard W, Holton B. 1998. The cyanobacterial toxin, microcystin-LR, can induce apoptosis in a variety of cell types. *Toxicon* **36**: 1981–1996.

Mikhailov A, Harmala-Brasken AS, Hellman J, Meriluoto J, Eriksson JE. 2003. Identification of ATP-synthase as a novel intracellular target for microcystin-LR. *Chem. Biol. Interact.* **142**: 223–237.

Milutinovic A, Sedmak B, Horvat-Znidarsic I, Suput D. 2002. Renal injuries induced by chronic intoxication with microcystins. *Cell. Mol. Biol. Lett.* **7**: 139–141.

Milutinovic A, Zivin M, Zorc-Pleskovic R, Sedmak B, Suput D. 2003. Nephrotoxic effects of chronic administration of microcystins -LR and -YR. *Toxicon* **42**: 281–288.

Miura GA, Robinson NA, Geisbert TW, Bostian KA, White JD, Pace JG. 1989. Comparison of *in vivo* and *in vitro* toxic effects of microcystin-LR in fasted rats. *Toxicon* **27**: 1229–1240.

Miura GA, Robinson NA, Lawrence WB, Pace JG. 1991. Hepatotoxicity of microcystin-LR in fed and fasted rats. *Toxicon* **29**: 337–346.

Moreno I, Pichardo S, Jos A, Gomez-Amores L, Mate A, Vazquez CM, Camean AM. 2005. Antioxidant enzyme activity and lipid peroxidation in liver and kidney of rats exposed to microcystin-LR administered intraperitoneally. *Toxicon* **45**: 395–402.

Nicholls DG. 1982. *Bioenergetics. An Introduction to the Chemiosmotic Theory*. Academic Press: London.

Nobre ACL, Coelho GR, Coutinho MCM, Silva MMM, Angelim EV, Menezes DB, Fonteles MC, Monteiro HSA. 2001. The role of phospholipase A(2) and cyclooxygenase in renal toxicity induced by microcystin-LR. *Toxicon* **39**: 721–724.

Nobre ACL, Coutinho MCM, Silva MMM, Fonteles MC, Monteiro HSA. 1998. Determination of the acute toxicity, study of the renal alterations and histopathologic evaluation of the effects of the microcystin. *Naunyn Schmiedebergs Arch. Pharmacol.* **358**: R441–R441.

Nobre ACL, Jorge MCM, Menezes DB, Fonteles MC, Monteiro HSA. 1999. Effects of microcystin-LR in isolated perfused rat kidney. *Braz. J. Med. Biol. Res.* **32**: 985–988.

Nobre ACL, Martins AMC, Havt A, Benevides C, Lima AAM, Fonteles MC, Monteiro HSA. 2003. Renal effects of supernatant from rat peritoneal macrophages activated by microcystin-LR: role protein mediators. *Toxicon* **41**: 377–381.

Nobre ACL, Nunes-Monteiro SM, Monteiro MCSA, Martins AMC, Havt A, Barbosa PSF, Lima AAM, Monteiro HSA. 2004. Microcystin-LR promote intestinal secretion of water and electrolytes in rats. *Toxicon* **44**: 555–559.

Oudra B, Loudiki M, Sbiyyaa B, Martins R, Vasconcelos V, Namikoshi N. 2001. Isolation, characterization and quantification of microcystins (heptapeptides hepatotoxins) in *Microcytis aeruginosa* dominated bloom of Lalla Takerkoust lake-reservoir (Morocco). *Toxicon* **39**: 1375–1381.

Pflugmacher S, Wiegand C, Oberemm A, Beattie KA, Krause E, Codd GA, Steinberg CEW. 1998. Identification of an enzymatically formed glutathione conjugate of the cyanobacterial hepatotoxin microcystin-LR: the first step of detoxication. *Biochim. Biophys. Acta General Subjects* **1425**: 527–533.

Pouria S, de Andrade A, Barbosa J, Cavalcanti RL, Barreto VTS, Ward CJ, Preiser W, Poon GK, Neild GH, Codd GA. 1998. Fatal microcystin intoxication in haemodialysis unit in Caruaru, Brazil. *Lancet* **352**: 21–26.

Rabergh CMI, Bylund G, Eriksson JE. 1991. Histopathological effects of microcystin-LR, a cyclic peptide toxin from the cyanobacterium (blue-green-alga) *Microcystis aeruginosa*, on common carp (*Cyprinus carpio* L). *Aquatic Toxicol.* **20**: 131–145.

Runnegar MTC, Falconer IR. 1982. The *in vivo* and *in vitro* biological effects of the peptide hepatotoxin from the blue-green-alga *Microcystis aeruginosa*. *S. Afr. J. Sci.* **78**: 363–366.

Runnegar MTC, Gerdes RG, Falconer IR. 1991. The uptake of the cyanobacterial hepatotoxin microcystin by isolated rat hepatocytes. *Toxicon* **29**: 43–51.

Soares RM, Yuan M, Servaites JC, Delgado A, Maglhaes VF, Hilborn ED, Carmichael WW, Azevedol SMFO. 2006. Sublethal exposure from microcystins to renal insufficiency patients in Rio de Janeiro, Brazil. *Environ. Toxicol.* **21**: 95–103.

Takenaka S. 2001. Covalent glutathione conjugation to cyanobacterial hepatotoxin microcystin LR by F344 rat cytosolic and microsomal glutathione S-transferases. *Environ. Toxicol. Pharmacol.* **9**: 135–139.

Wickstrom ML, Khan SA, Haschek WM, Wyman JF, Eriksson JE, Schaeffer DJ, Beasley VR. 1995. Alterations in microtubules, intermediate filaments, and microfilaments induced by microcystin-LR in cultured-cells. *Toxicol. Pathol.* **23**: 326–337.

Yoshida T, Makita Y, Tsutsumi T, Nagata S, Tashiro F, Yoshida F, Sekijima M, Tamura S, Harada T, Maita K, Ueno Y. 1998. Immunohistochemical localization of microcystin-LR in the liver of mice: A study on the pathogenesis of microcystin-LR-induced hepatotoxicity. *Toxicol. Pathol.* **26**: 411–418.

Zegura B, Lah TT, Filipic M. 2004. The role of reactive oxygen species in microcystin-LR-induced DNA damage. *Toxicology* **200**: 59–68.

Zoratti M, Szabò I. 1995. The mitochondrial permeability transition. *Biochim. Biophys. Acta* **1241**: 139–176.

DOI: 10.1002/jat