

Microcystin-LR acute exposure does not alter *in vitro* and *in vivo* ATP, ADP and AMP hydrolysis in adult zebrafish (*Danio rerio*) brain membranes

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Abstract

Microcystins (MCs) are toxins produced by cyanobacteria during the blooms that could accumulate in aquatic animals and be relocated to higher trophic levels. Adenosine triphosphate (ATP) acts as an excitatory neurotransmitter and/or a neuromodulator in the extracellular space playing important roles in physiological and pathological conditions. The aim of this study was, therefore, to evaluate the acute effects of different concentrations of MC-LR on nucleoside triphosphate diphosphohydrolases and 5'-nucleotidase in adult zebrafish (*Danio rerio*) brain membranes. The results have shown no significant changes in ATP, adenosine diphosphate (ADP) and adenosine monophosphate (AMP) hydrolysis in zebrafish brain membranes. MC-LR *in vitro* also did not alter ATP, ADP and AMP hydrolysis in the concentrations tested. These findings show that acute exposure to MC-LR did not modulate ectonucleotidase activity in the conditions tested. However, additional studies including chronic exposure should be performed in order to achieve a better understanding about MC-LR toxicity mechanisms in the central nervous system.

Introduction

Microcystins (MCs) are heptapeptides primarily known as potent hepatotoxins released into water during cyanobacterial blooms.¹ More recently, it was demonstrated that MCs are also potential neurotoxic compounds.²⁻⁴ MCs inhibit serine/threonine-specific protein phosphatases (PPs) such as PP1 and PP2A, induce oxidative stress, and generate cell apoptosis.⁵

Adenosine triphosphate (ATP) is an important signaling molecule in the extracellular space which plays key roles in physiological and pathological conditions. Once released into the extracellular space, the ATP can be metabolized by the action of ectoenzymes that convert this nucleotide to adenosine.⁶ The ATP and metabolites, including adenosine diphosphate (ADP) and adenosine, affect several crucial biological events, including neurotransmission and neuromodulation, platelet aggregation, heart contraction,⁷ the normal and abnormal cell growth and apoptosis,⁸ and intracellular signaling.⁹

Nucleoside triphosphate diphosphohydrolases (NTPDases) hydrolyze triphosphonucleosides and diphosphate with different abilities. The NTPDase1 (CD39) hydrolyzes ATP and ADP in the same way (1:1) while NTPDase2 (CD39L1) prefers trifosfonucleosídeos (in a ratio of 30:1), known as ecto-ATPase. The NTPDase3 (CD39L3, HB6) and NTPDase8 prefer ATP to ADP at a ratio of 3:1 and 2:1, respectively. Other members of the NTPDase family are associated with membranes of intracellular organelles (NTPDase4-7). Ecto-5'-nucleotidase is the enzyme responsible for adenosine monophosphate (AMP) hydrolysis, generating adenosine, the final product of ATP breakdown.⁶

Zebrafish (*Danio rerio*) is a small teleost that has long been considered a successful animal model for studying diverse biological actions. More recently, zebrafish has also become a valuable model for toxicological and neurochemical studies, including those performed to investigate the toxic effects generated by MC-LR exposure in the liver^{10,11} and in the brain.^{2-4,12}

Wang and colleagues² demonstrated that MC-LR causes neurotoxicity in zebrafish at the proteomic level. MC-LR induced oxidative stress, a dysfunction of cytoskeleton assembly, and macromolecule metabolism. In agreement with this, Meng and colleagues³ showed that MC-LR leads to the reorganization of cytoskeletal architectures in the neuroendocrine (PC12) cells through hyperphosphorylation of tau and HSP27 via PP2A inhibition and subsequent activation of the p38 MAPK signaling pathway. In addition, MC-LR acute exposure increased acetylcholinesterase

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(AChE) activity via transcriptional *ache* activation in zebrafish brain⁴ and altered adult zebrafish swimming performance parameters.¹² This study was designed in response to a series of factors: i) MCs synthesized by cyanobacteria can either accumulate in the brain and change locomotor behavior in fish; ii) the neurotoxic effects of MCs exposure are far from being completely understood; iii) zebrafish is a well-established organism model for toxicological analysis; and iv) the purinergic system has been extensively studied in this species. Therefore, the objective of this study was to investigate whether MC-LR acute exposure was able to modulate *in vitro* and *in vivo* ATP, ADP and AMP hydrolysis in the brain of adult zebrafish, and promote a better understanding of the complete scenario of MC-LR toxicity.

Materials and Methods

Animals

Wild-type adult (<8 months old) zebrafish (*Danio rerio*) of both sexes (120 animals for *in vivo* exposure and 50 animals for *in vitro* analysis) were obtained from a specialized supplier (Redfish Agroloja, RS, Brazil). Animals were kept in 50 L housing tanks with tap water previously treated with Tetra's AquaSafe® (to neutralize chlorine, chloramines, and heavy metals present in the water that could be harmful to fish) and continuously aerated (7.20 mg O₂/L) at 26±2°C, under a 14-10 h light/dark photoperiod, a maximum of 5 animals per liter. Animals were acclimated for at least two weeks before the experiments. They were fed three times a day with TetraMin Tropical Flake fish®.

The procedures were previously approved by the Animal Ethics Committee of Pontifical Catholic University of Rio Grande do Sul (PUCRS) under the protocol number 10/00142-CEUA.

Reagents

Microcystin-LR was purchased from BioVision (Milpitas, CA, USA). Trizma base, malachite green, ammonium molybdate, polyvinyl alcohol, ethylene diamine tetraacetic acid (EDTA), ethylene glycol tetraacetic acid (EGTA), sodium citrate, Coomassie Blue G, bovine serum albumin, calcium chloride, magnesium chloride, and nucleotides (ATP, ADP and AMP) were purchased from the Sigma Chemical Co. (St. Louis, MO, USA). All other reagents used were analytical grade.

In vivo exposure

In vivo exposures were performed in 1-L aquariums (10 fish per aquarium). Fish were exposed to different MC-LR concentrations (50 and 100 µg/L) dissolved in water for 24 h. Control group animals were exposed only to water for 24 h, after which they were euthanized by decapitation.

In vitro exposure

In vitro assays were performed as previously described.¹³ Briefly, MC-LR was added to the reaction medium before the pre-incubation with zebrafish brain membranes and maintained during the enzyme assays. MC-LR was tested at a final concentration of 50, 100, 500, and 1000 µg/L. For the control group, the enzyme assay was performed in the absence of MC-LR (*i.e.* no toxin was added to the reaction medium).

Membrane preparation

Brain membranes were prepared as described previously.¹⁴ Zebrafish were eutha-

nized by decapitation and their whole brains were removed from the cranial skull by the dissection technique. For each sample (membrane preparation), a pool of 5 zebrafish brains was used. These were briefly homogenized in 60 volumes (v/w) of chilled Tris-citrate buffer (50 mM Tris, 2 mM EDTA, 2 mM EGTA, pH 7.4, with citric acid) in a motor driven Teflon-glass homogenizer. The samples were centrifuged at 1000 g for 10 min and the pellet was discarded. The supernatant was then centrifuged for 25 min at 40,000 g. The resultant pellet was frozen in liquid nitrogen, thawed, resuspended in Tris-citrate buffer, and centrifuged for 20 min at 40,000 g. This freeze-thaw-wash procedure was used to ensure the lysis of the brain membranes. The final pellet was resuspended and used in the enzyme assays. All samples were maintained at 2-4°C throughout preparation.

Nucleotide hydrolysis assay

Zebrafish brain membranes (3 µg protein for NTPDase and 5 µg protein for ecto-5'-nucleotidase) were added to the reaction mixture containing 50 mM Tris-HCl (pH 8.0) and 5 mM CaCl₂ (for NTPDase activity) or 50 mM Tris-HCl (pH 7.2) and 5 mM MgCl₂ (for ecto-5'-nucleotidase activity) in a final volume of 200 µL. The samples were pre-incubated for 10 min at 37°C and the reaction was initiated by the addition of substrate (ATP, ADP, AMP) to a final concentration of 1 mM. The reaction was stopped after 30 min with the addition of 200 µL of 10% trichloroacetic acid and immediately placed on ice for 10 min. The inorganic phosphate (Pi) released was determined by colorimetric assay.¹⁵ Each sample was mixed to 250 µL of Malachite Green solution and the nucleotide hydrolysis was measured in a microplate reader (Thermoplate-TP Reader) at 630 nm after 20 min. Controls with the addition of the enzyme preparation after the incubation period were used to correct non-enzymatic hydrolysis of substrates. Incubation times and protein concentrations were chosen to ensure the linearity of the reactions. Specific activity was expressed as nanomoles of Pi released per minute per milligram of protein. All enzyme assays were run in duplicate of at least 4 independent experiments.

Protein determination

Protein was measured by the Coomassie blue method¹⁶ using bovine serum albumin as standard.

Statistical analysis

Data were expressed as means±SEM (standard error mean). The activity of ectonucleotidases for each substrate (ATP,

ADP and AMP) was assessed by one-way analysis of variance (one-way ANOVA).

Results

In this study, we analyzed the effects of distinct MC-LR concentration exposure on ectonucleotidases activities (NTPDases and 5'-nucleotidase) by performing *in vivo* (50 and 100 µg/L) and *in vitro* (50, 100, 500 and 1000 µg/L) experiments using adult zebrafish. The *in vivo* analysis demonstrated that ectonucleotidases activities were not altered in the animals treated with different concentrations ($P>0.05$; Figure 1).

To verify whether the toxin might have a direct effect on the enzymes of purinergic signaling, we tested the *in vitro* effect of MC-LR on ectonucleotidase activities in zebrafish brain membranes. The results showed that MC-LR did not cause any alteration in NTPDases and 5'-nucleotidase activities in the conditions tested ($P>0.05$; Figure 2).

Discussion

Recently, the cholinergic system was demonstrated to be a target to acute exposition to MC-LR in adult zebrafish brain.⁴ Acetylcholine (ACh), the main neurotransmitter of cholinergic system, is co-released together with ATP at synaptic cleft¹⁷ and studies have demonstrated that ATP can control the ACh release.¹⁸ In addition, several studies have already demonstrated that toxic compounds to brain such as methanol¹⁹ and ethanol,^{20,21} affected both cholinergic and purinergic neurotransmission systems. For these reasons, we investigated the *in vivo* and *in vitro* effects of MC-LR acute exposure over NTPDases and 5'-nucleotidase activities on adult zebrafish brain membranes.

Results demonstrate that ATP, ADP and AMP hydrolysis *in vivo* were not altered by the action of MC-LR in any concentration tested on adult zebrafish brain membranes. To our knowledge, the possible toxic effects caused by MC-LR exposure on the enzymes involved in purinergic neurotransmission have not yet been investigated in any organism. In addition, a possible direct effect of MC-LR on these enzymes activities was also evaluated. Our results showed that MC-LR did not directly alter NTPDases and 5'-nucleotidase activities on zebrafish brain membranes. These results are in accordance with a previous study in which MC-LR did not affect *in vitro* AChE activity.⁴ It is possible to speculate that the absence of effects over

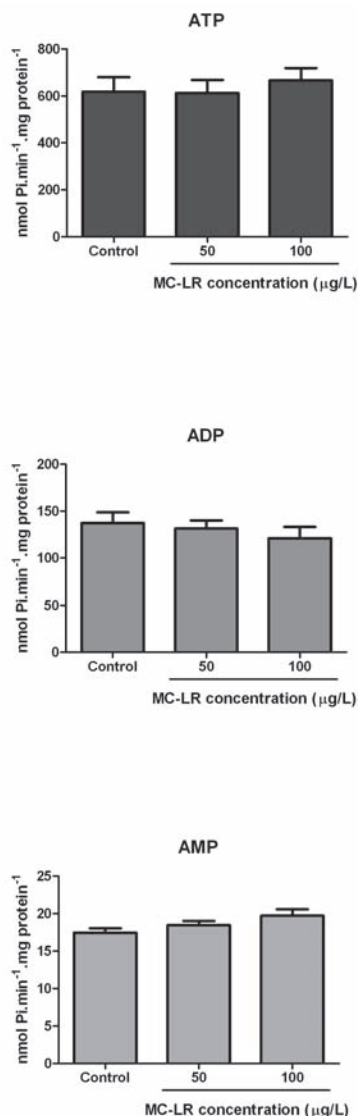


Figure 1. *In vivo* effect of acute treatment with microcystins-LR on (A) adenosine triphosphate (ATP), (B) adenosine diphosphate (ADP) and (C) adenosine monophosphate (AMP) hydrolysis in zebrafish brain membranes. Bars represent the mean±SEM. Data were analyzed by one-way ANOVA, $P < 0.05$. The specific enzyme activity is reported as nanomole of inorganic phosphate released per minute per milligram of protein.

ATP, ADP and AMP hydrolysis on the brain of zebrafish after MC-LR exposure could be a consequence of the strategy adopted, *i.e.* acute exposure (24 h) and the concentrations of the toxin tested (50 and 100 g/L). Further studies including long-time exposure and higher concentrations of the toxin should be performed to achieve a better understanding about MC-LR toxicity mechanisms in the central nervous system.

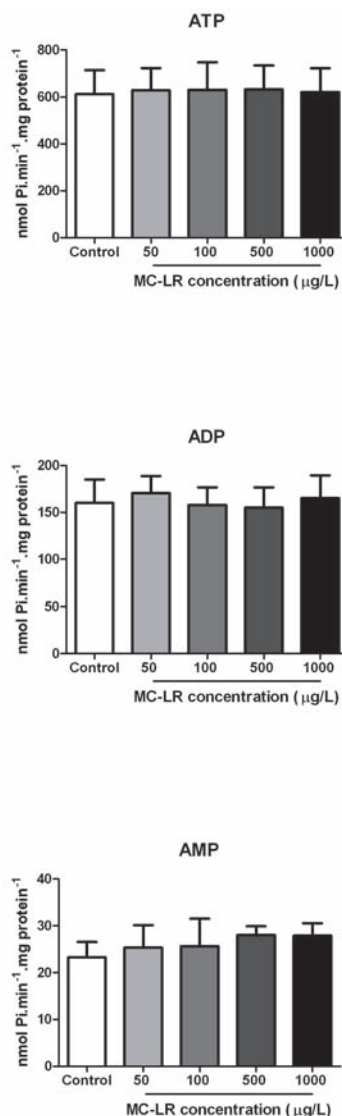


Figure 2. *In vitro* effect of distinct concentrations of microcystins-LR on (A) adenosine triphosphate (ATP), (B) adenosine diphosphate (ADP) and (C) adenosine monophosphate (AMP) hydrolysis in zebrafish brain membranes. Bars represent the mean±SEM. Data were analyzed by one-way ANOVA, $P < 0.05$. The specific enzyme activity is reported as nanomole of inorganic phosphate released per minute per milligram of protein.

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