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

Luiza Wilges Kist,[1,2] Rachel Seemann Fritsch,[1] João Sarkis Yunes,[3] Carla Denise Bonan,[2,4] Maurício Reis Bogo[1,2]

[1]Laboratório de Biologia Genômica e Molecular, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS; [2]Instituto Nacional de Ciência e Tecnologia Translacional em Medicina (INCT-TM), Porto Alegre, RS; [3]Unidade de Pesquisas em Cianobactérias, Prédio da Hidroquímica, Instituto de Oceanografia, Campus Carreiros da FURG; [4]Laboratório de Neuroquímica e Psicofarmacologia, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil

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'-nucleotidade 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 colleagues[2] 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 colleagues[3] 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 (AChE) activity via transcriptional *ache* activation in zebrafish brain[4] 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.

Correspondence: Maurício Reis Bogo, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Avenida Ipiranga, 6681 - 12C - sala 134, 90619-900, Porto Alegre, RS, Brazil.
Tel. +55.51.3353.4726 - Fax: +55.51.3320.3568.
E-mail address: mbogo@pucrs.br

Key words: microcystin-LR, zebrafish, ectonucleotidases, neurotoxicity.

Acknowledgments: this work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) (Proc. 472298/2010-0) and DECIT/SCTIEMS through CNPq and Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS) (Proc. FAPERGS 10/0036-5 - PRONEX). LWK was recipient of fellowship from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES). RSF was recipient of fellowship from CNPq. The authors thank Fernanda C. Vuaden for technical support.

Contributions: LWK and MRB study conception and design, data acquisition, analysis and interpretation, article drafting and revision; RSF data acquisition and article revision; JSY and CDB study conception and design, and article revision. All authors approved the final version to be published.

Conflict of interests: the authors declare no conflict of interests.

Received for publication: 5 July 2012.
Revision received: 3 September 2012.
Accepted for publication: 11 September 2012.

Journal of Xenobiotics 2012; 2:e9
doi:10.4081/xeno.2012.e9

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 O_2/L) at 26±2ºC, 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 euthanized 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 $CaCl_2$ (for NTPDase activity) or 50 mM Tris-HCl (pH 7.2) and 5 mM $MgCl_2$ (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 method[16] 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 cleft[17] 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 methanol[19] 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

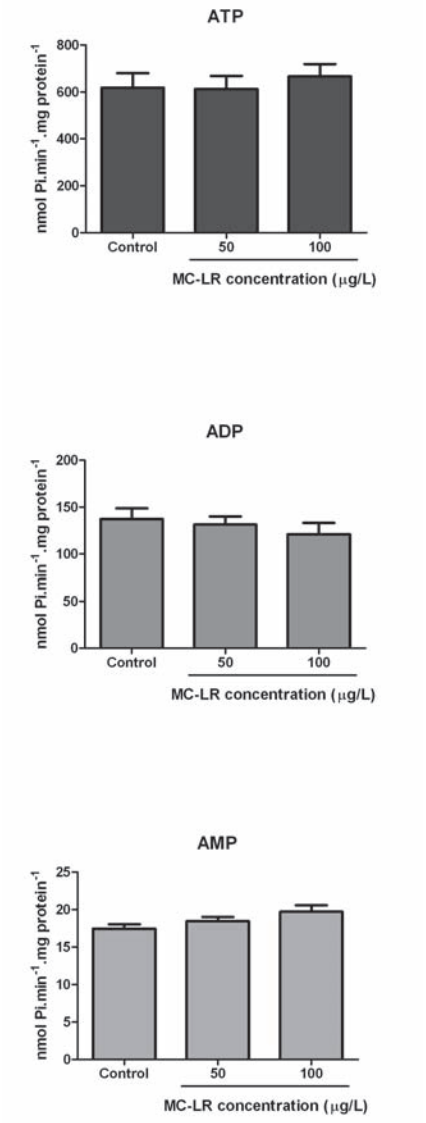

Figure 1. ***In vivo*** **effect of acute treatment with microcystins-LR on (A) adenosine triphosphate (ATP), (B) adenosine diphosphate (ADP) and (C) adenosine monophosphate (AMP) hydrolysis in zebrafish brain membranes. Bars represent the mean±SEM. Data were analyzed by one-way ANOVA, P<0.05.The specific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

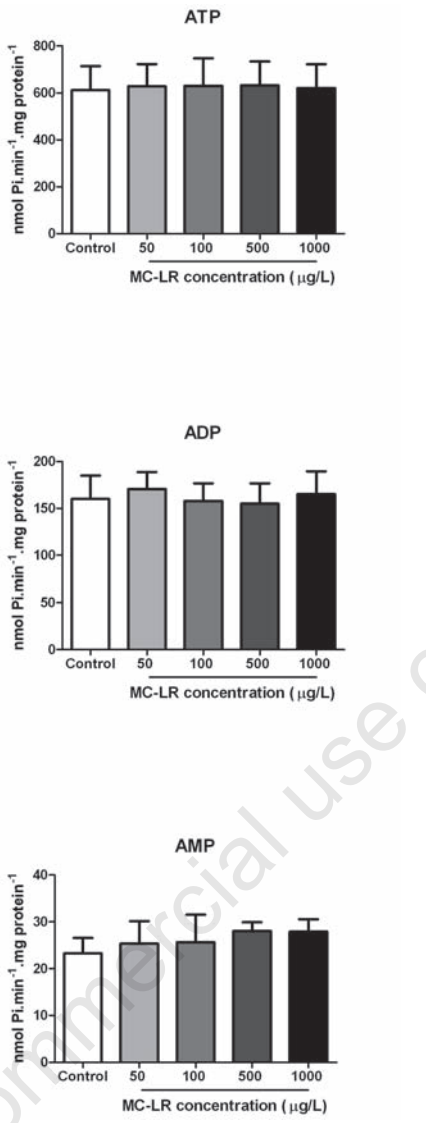

Figure 2. ***In vitro*** **effect of distinct concentrations of microcystins-LR on (A) adenosine triphosphate (ATP), (B) adenosine diphosphate (ADP) and (C) adenosine monophosphate (AMP) hydrolysis in zebrafish brain membranes. Bars represent the mean±SEM Data were analyzed by one-way ANOVA, P<0.05.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

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.

References

1. Malbrouck C, Kestemont P. Effects of microcystins on fish. Environ Toxicol Chem 2006;25:72-86.
2. Wang M, Wang D, Lin L, Hong H. Protein profiles in zebrafish (*Danio rerio*) brains exposed to chronic microcystin-LR. Chemosphere 2010;81:716-24.
3. Meng G, Sun Y, Fu W, Guo Z, Xu L. Microcystin-LR induces cytoskeleton system reorganization through hyperphosphorylation of tau and HSP27 via PP2A inhibition and subsequent activation of the p38 MAPK signaling pathway in neuroendocrine (PC12) cells. Toxicology 2011;290:218-29.
4. Kist LW, Rosemberg DB, Pereira TC, de Azevedo MB, Richetti SK, de Castro Leão J, et al. Microcystin-LR acute exposure increases AChE activity via transcriptional ache activation in zebrafish (*Danio rerio*) brain. Comp Biochem Physiol C Toxicol Pharmacol 2012;155:247-52.
5. Campos A, Vasconcelos V. Molecular mechanisms of microcystin toxicity in animal cells. Int J Mol Sci 2010;11:268-87.
6. Zimmermann H. Ectonucleotidases: some recent developments and a note on nomenclature. Drug Dev Res 2001;52:44-56.
7. Ralevic V, Burnstock G. Receptors for purines and pyrimidines. Pharmacol Ver 1998;50:413-92.
8. Goepfert C, Imai M, Brouard S, Csizmadia E, Kaczmare K, Robson S. CD39 modulates endothelial cell activation and apoptosis. Mol Med 2000;6:591-603.
9. Schlosser SF, Burgstahler AD, Nathanson JA. Isolated rat hepatocytes can signal to other hepatocytes and bile duct cells by release of nucleotides. Proc Natl Acad Sci USA 1996;93:9948-53.
10. Wei L, Sun B, Song L, Nie P. Gene expression profiles in liver of zebrafish treated with microcystin-LR. Environ Toxicol Pharmacol 2008;26:6-12.
11. Ji W, Liang H, Zhou W, Zhang X. Apoptotic responses of zebrafish (*Danio rerio*) after exposure with microcystin-LR under different ambient temperatures. J Appl Toxicol 2012. [Epub ahead of print].
12. Kist LW, Piato AL, da Rosa JG, Koakoski G, Barcellos LJ, Yunes JS, et al. Acute Exposure to microcystin-producing cyanobacterium microcystis aeruginosa alters adult zebrafish (*Danio rerio*) swimming performance parameters. J Toxicol 2011;2011:280304.
13. Siebel AM, Rico EP, Capiotti KM, Piato AL, Cusinato CT, Franco TM, et al. *In vitro* effects of antiepileptic drugs on acetylcholinesterase and ectonucleotidase activities in zebrafish (*Danio rerio*) brain. Toxicol In Vitro 2010;24:1279-84.
14. Barnes JM, Murphy PA, Kirkham D, Henley JM. Interaction of guanine nucleotides with [3H]kainate and 6-[3H]cyano-7-nitroquinoxaline-2,3-dione binding in goldfish brain. J Neurochem 1993;61:1685-91.
15. Chan KM, Delfert D, Junger KD. A direct colorimetric assay for Ca2+-stimulated

ATPase activity. Anal Biochem 1986;157:375-80.
16. Bradford MM. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976;72:218-54.
17. Burnstock G. Cotransmission. Curr Opin Pharmacol 2004;4:47-52.
18. Cunha RA, Ribeiro JA. ATP as a presynaptic modulator. Life Sci 2000;68:119-37.
19. Rico EP, Rosemberg DB, Senger MR, Arizi M de B, Bernardi GF, Dias RD, et al. Methanol alters ecto-nucleotidases and acetylcholinesterase in zebrafish brain. Neurotoxicol Teratol 2006;28:489-96.
20. Rico EP, Rosemberg DB, Dias RD, Bogo MR, Bonan CD. Ethanol alters acetylcholinesterase activity and gene expression in zebrafish brain. Toxicol Lett 2007;174:25-30.
21. Rico EP, Rosemberg DB, Senger MR, de Bem Arizi M, Dias RD, Souto AA, et al. Ethanol and acetaldehyde alter NTPDase and 5'-nucleotidase from zebrafish brain membranes. Neurochem Int 2008;52:290-6.