Study on apoptotic effects of neurotoxin anatoxin-a on fish immune cells

Anna Rymuszka, Anna Sierosławska

Department of Physiology and Ecotoxicology, Institute of Biotechnology,
The John Paul II Catholic University of Lublin, Poland

Correspondence to: Anna Rymuszka, PhD.
Department of Physiology and Ecotoxicology, Institute of Biotechnology,
The John Paul II Catholic University of Lublin,
14 Al. Racławickie str, 20–950 Lublin, Poland.
TEL: +48 81 53 337 84; FAX: +48 81 53 670 89; E-MAIL: anrym@kul.lublin.pl

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Abstract

OBJECTIVES: The aim of this study was to assess the possible *in vitro* apoptotic effects of anatoxin-a on the selected immune cells isolated from the blood of carp.
DESIGN: In the experiments pure anatoxin-a was used at concentrations of 0.01, 0.05, 0.1 and 1 μg/ml RPMI–1640 medium. Apoptosis or necrosis of fish leukocytes (lymphocytes and phagocytes) induced by the toxin was determined by measurement of the activity of caspases-3/7 and the analysis of phosphatidylserine on the outer leaflet of apoptotic cell-membranes using Annexin-V-Fluorescein and Propidium Iodide. Moreover, fluorescent measurement of the release of lactate dehydrogenase from the cells with damaged membranes was done.
RESULTS: The viability of the lymphocytes exposed only to the highest concentration of anatoxin-a (1 μg/ml) was significantly decreased. The exposure to the toxin at higher concentrations (0.1 and 1 μg/ml) resulted in a significant increase of caspases 3/7 activity in phagocytes and lymphocytes. Moreover, fluorescent analysis with the use of annexin-V-fluorescein and propidium iodide staining showed more cells at the apoptotic stage than necrotic cells.
CONCLUSION: Our study showed that anatoxin-a is an inducer of apoptosis in fish immune cells.

Abbreviations:

ANTX-a	-anatoxin-a
LDH	- lactate dehydrogenase
nAChRs	- nicotinic acetylcholine receptors
PI	- propidium iodide

ORIGINAL ARTICLE

INTRODUCTION

Widespread water eutrophication causes massive development of cyanobacteria (phytoplanktonic organisms) often forming blooms in many reservoirs: lakes and recreational waters. Some of these blooms are able to produce various, highly toxic secondary metabolites-cyanotoxins, among which are potent hepatotoxins, neurotoxins, cytotoxins, dermatoxins and tumor promoters (Wiegand & Pflugmacher 2005; Sierosławska & Rymuszka 2009).

ANTX-a (anatoxin-a) is a naturally occurring homotropane alkaloid isolated from toxic strains of freshwater cyanobacteria including *Anabaena flos-aquae, Anabaena planktonia*, *Aphanizomenon flos-aquae*, *Cylindrospermum* sp. and *Microcystis* species (Carmichael *et al.* 1975; Osswald *et al.* 2007b).

ANTX-a is a neurotoxin, an agonist of acetylcholine binding the nicotinic receptors (nAChRs). Since the toxin is not degraded by cellular enzymes, it induces severe overstimulation of neurons. High doses of ANTX-a may cause death by respiratory arrest (Aronstam & Witkop 1981; Swanson *et al.* 1986; Rogers *et al.* 2006). Poisoning episodes caused by this toxin, of wild and domestic animals in North America and in Europe were found in recent years (Edwards *et al.* 1992; Hamill *et al.* 2001; Gugger *et al.* 2005).

Although these cyanobacterial genera are found commonly in the world and they frequently create water blooms, there are few reported studies of the influence ANTX-a on fish (Oberemm *et al.* 1999; Osswald *et al.* 2007a; Osswald *et al.* 2009; Rymuszka & Sierosławska 2009). During a period of a few years ANTX-a has been identified in a highly eutrophic dam reservoir, Zemborzycki, near Lublin (SE Poland) in spring and summer warm seasons (Pawlik-Skowrońska *et al.* 2004; Sierosławska *et al.* 2010). The purpose of this study was to investigate whether the cyanotoxin, ANTX-a induces apoptosis or necrosis in fish lymphocytes and phagocytes.

MATERIAL AND METHODS

Experimental fish

Carp (*Cyprinus carpio* L) weighing 100–200 g were obtained from a commercial farm and maintained in 80L tanks (6–7 fish in each tank) under a natural photoperiod. The research was approved by the guidelines for the care of laboratory animals (Local Committee of Ethics, approval number 9/2009).

Cell separation

The cell suspensions of blood were prepared according to the technique from Rowley (1990) with slight modifications. Briefly, blood was taken from the caudal vein, diluted 1:1 in phosphate buffered saline (PBS, Biomed, Lublin, Poland) containing 20 IU/ml heparin (Polfa, Warsaw, Poland). The diluted blood was placed on discontinuous Percoll gradients (Sigma) and centrifuged for 40 min at 400 × g and 4 °C. Leucocyte-rich fractions were collected from the interfaces of the two Percoll densities (1.07 g/ml for phagocytes, 1.02 g/ml for lymphocytes) and washed twice with RPMI-1640 containing 10% fetal calf serum (Gibco) supplemented with 1% penicillin–streptomycin (Sigma) (complete RPMI-1640). Phagocytes isolated from blood included mainly neutrophils (90%) and monocytes (10%). The cells were counted and their viability was determined using a Nucleo Counter YC-100 (Chemometec) according to the manufacturer's procedure. Blood leucocyte viability found to be >95%. The cells were adjusted to required density for each test.

LDH assay

The CytoTox-One™ Homogeneous Membrane Integrity Assay (Promega, Madison, WI, USA) with fluorescence readout was used to measure LDH activity. Briefly, the experiment was performed with control (unexposed cells), lysis control (cells treated with 1% Triton X-100; determinate total cellular LDH activity) and experimental groups (cells exposed to ANTX-a at the concentrations from 0.01 to 1 μg/ml). After 24h incubation with the toxin 100 μl of cell suspension (2×10^4 cells/ml) was transferred to the black 96-well plate and 100 μl of Cyto-Tox-ONE reagent was added to each well. The plates were incubated for 10 min at room temperature in the dark. The reaction was stopped by addition of 50 μl of Stop solution. Total cellular LDH activity was measured after the cells were lysed prior to the reagent addition. The plate was shaken for 10 seconds and fluorescence was measured using a fluorometer (FLUOstar Optima, BMG Labortechnik) with excitation wavelength of 560 nm and an emission wavelength of 590 nm.

Caspase activity assay

The activity of caspases 3 and 7 was measured by cleavage of their specific substrate which leads to a measurable fluorometric colour reaction. Cells were seeded at 10^4 cells per well into white 96-well plates and exposed to ANTX-a at 0.01, 0.05, 0.1 and 1 μg/ml for 24 h at room temperature. At the end of the incubation period, 100 μl of Apo-ONE Caspase-3/7 reagent was added to wells followed by the procedure recommended by the manufacturer. The suspension in the wells was mixed gently and incubated for 1 h in the dark at room temperature. Fluorescence was measured at 485 nm (excitation) and 520 nm (emission) with the fluorescence microplate reader (FLUOstar Optima, BMG LABTECH, Germany).

Annexin-V staining

Phosphatidylserine translocation was used to determine cell apoptosis. Detection of plasma membrane alterations in cells exposed to ANTX-a was performed using the Annexin-V-FLUOS staining kit (Roche, Mannheim, Germany) according to the manufacturer's

protocol. The cells (1 × 10^6 cells/ml) were incubated in 8-well chamber slides with or without ANTX-a at concentration 1 µg/ml for 24 h at room temperature. After incubation, the experimental medium was aspirated and washed with PBS, and cells were resuspended in Annexin-V-fluorescein in a Hepes buffer containing propidium iodide (PI) for 15 min at room temperature in the dark. The slides were directly analyzed by fluorescence microscopy at 400 × magnification (Nicon Exlipse 80i). The cells stained only with Annexin-V-FLUOS (green color), interacting with phosphatidylserine on the outer plasma membrane, were interpreted as undergoing early apoptosis. PI (red color), enters the cell and interacts with the nuclear DNA and RNA molecules in the nucleus and cytosol regions in cells. The double-colour stained cells were considered late apoptotic (Annexin-V- FLUOS+ PI) or necrotic.

Statistical analysis

All values were presented as mean±S.D (Standard Deviation). One-way ANOVA (analysis of variance) followed by the Duncan`s test was performed for statistical comparisons; a *p*-value of less than 0.05 was considered as significant.

RESULTS

LDH assay

ANTX-a at concentrations of 0.01, 0.05 and 0.1 µg/ml reduced the viability of the cells to less than 10% (Figure 1). Only at the highest concentration of toxin the statistically significant ($p \leq 0.05$) release of LDH from lymphocytes with a damaged membrane was recorded.

Caspase activity assay

Incubation of lymphocytes and phagocytes with the toxin at higher concentrations (0.1 and 1 µg/ml) resulted in a significant ($p \leq 0.05$) increase in caspase 3/7 activity as compared to the unexposed cells in controls (Figure 2).

Annexin-V staining

To determine if the toxin induces either apoptotic or necrotic cell death, annexin-V-FLUOS and PI staining method were used. In the early stages of apoptosis, phosphatidylserine translocates from the inner part of the plasma membrane to the outer layer, exposing it on the external surface of the cell. No significant green fluorescence or PI staining (red color) was observed in control. As shown in Figure 3, after incubation of the immune cells with the neurotoxin at the concentration of 1 µg/ml for 24 h, more cells were at the apoptotic stage and less cells (with green and red labeling) were at the necrosis or the late stage of apoptosis.

DISCUSSION

The occurrence and chemical properties of ANTX-a have been studied but their toxic effects on fish physiological functions, particularly immune system are still unknown. The main known toxic effect of this alkaloid is the neurotoxicity which may induce many abnormalities in animal behaviour with consequences for reproduction and changes in coordination. Experimental studies performed by Osswald *et al.* (2007a) showed that the exposure of juvenile carps for 4 days to freeze-dried cells of ANTX-a positive *Anabaena* sp strain induce abnormal swimming. Neurotoxic activity of toxin may lead to a temporal change of the heart rate in zebrafish embryos (Oberemm *et al.* 1999). Moreover, only higher concentrations of pure ANTX-a (640 µg/l) and extracts containing toxin (333 µg/l) showed to be toxic to early developmental stages of common carp (Osswald *et al.* 2009).

ANTX-a is one of the most toxic of known cyanotoxins. It is also a stress factor for the cells. There are only a few reports regarding the effects of ANTX-a on other types of cells than neurons. The study performed by Teneva and collaborators (2005) showed that the toxin caused cytotoxicity in cultured mouse T and B lymphocytes. In the present study, *in vitro* cytotoxicity of ANTX-a was measured by determination of

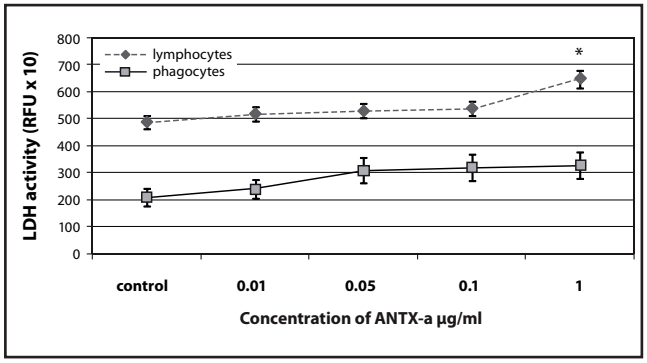

Fig. 1. *In vitro* effects of ANTX-a on blood phagocyte and lymphocyte viability (mean ± SD, n=10, *differences statistically significant when $p<0.05$)

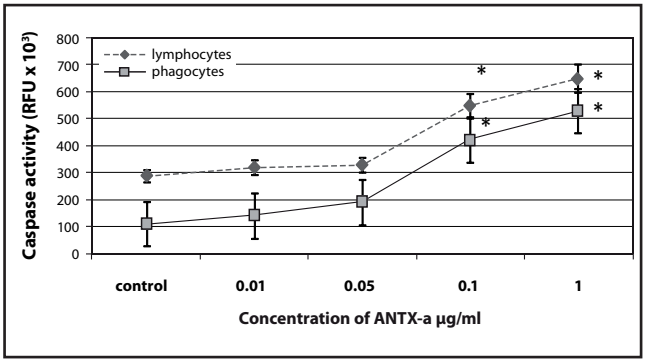

Fig. 2. Effect of ANTX-a on caspases-3/7 activity in fish phagocytes and lymphocytes. Caspases-3/7 activity was assayed 24 h after exposure to various concentrations of toxin (0.01, 0.05, 0.1 and 1 µg/ml). Data are shown as mean ± SD, n = 5, *differences statistically significant when $p<0.05$

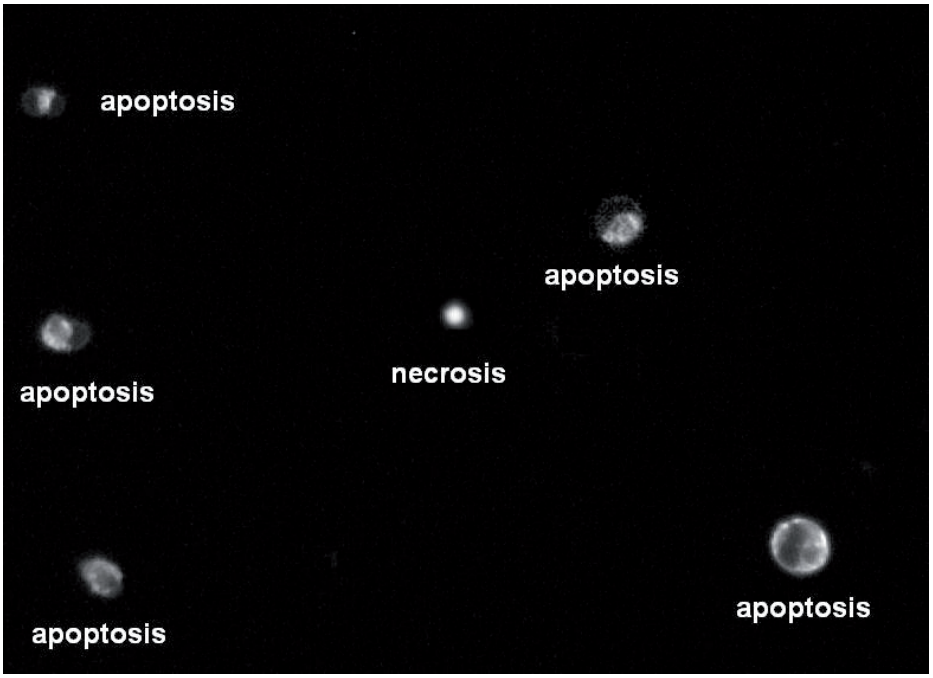

Fig. 3. The example image of staining Annexin-V and PI showning cell apoptosis and cell necrosis in carp immune cells exposed to ANTX-a at 1 µg/ml for 24 h. Photographs taken at a magnification of 400×. Data shown are representative of two independent experiments. The fluorescent stainings were measured by microscopic observation in at least 100 cells/ sample per slide.

the release of LDH as an indicator of cell membrane damage. Our results showed that the percent of LDH leakage was significantly altered only after the incubation of lymphocytes with the highest concentration of the toxin (Figure 1). The LDH release assay may be sensitive only to the highest, lytic concentrations of toxic factors that directly damage the cell membrane.

This neurotoxin may affect physiology of cells in different ways. Exposure to the toxin may induce oxidative stress, which can lead to structural and functional dysfunction by attacking chemical bonds in cellular lipids, proteins, and nucleic acids. The increase of ROS production can induce the activation of the caspase cascade, a key event of apoptotic process (Desagher & Martinou 2000). ANTX-a may cause oxidative stress in normal cells and further increase ROS levels in cells undergoing apoptosis. Our results on apoptosis of immune cells induced by the neurotoxin were in accordance with this hypothesis. A dose-dependent significant increase in the caspase-3/7 activity in immune cells was detected (Figure 2). The activation of this protein is an important step in apoptosis and is responsible for a majority of the morphological changes in apoptotic cell death (Krysko *et al.* 2008). Significant correlation was observed between the 3/7 caspases activity and presence of apoptotic changes in the immune cells incubated with the toxin for 24 h, as evidenced by the images of staining (Figure 3). ANTX-a caused higher proportions of apoptotic cells and lower proportions of necrotic cells. The study performed by Rao *et al.* (2002) also demonstrated that ANTX-a induced apoptosis in rat thymocytes and monkey kidney cells possibly by generation of reactive oxygen species and caspase activation.

In conclusion, the study has shown that neurotoxin ANTX-a induces apoptosis of fish immune cells.

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