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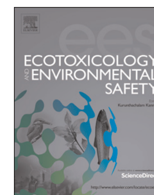

Proteomic analysis of anatoxin-a acute toxicity in zebrafish reveals gender specific responses and additional mechanisms of cell stress

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ABSTRACT

Anatoxin-a is a potent neurotoxin produced by several genera of cyanobacteria. Deaths of wild and domestic animals due to anatoxin-a exposure have been reported following a toxic response that is driven by the inhibition of the acetylcholine receptors at neuromuscular junctions. The consequent neuron depolarization results in an overstimulation of the muscle cells. In order to unravel further molecular events implicated in the toxicity of anatoxin-a, a proteomic investigation was conducted. Applying two-dimensional gel electrophoresis (2DE) and MALDI-TOF mass spectrometry, we report early proteome changes in brain and muscle of zebrafish (*Danio rerio*) caused by acute exposure to anatoxin-a. In this regard, the test group of male and female zebrafish received an intraperitoneal (i.p.) injection of an anatoxin-a dose of 0.8 $\mu g\ g^{-1}$ of fish body weight (bw) in phosphate buffered saline solution (PBS), while the control received an i.p. injection of PBS only. Five minutes after i.p. injection, brain and muscle tissues were collected, processed and analyzed with 2DE. Qualitative and quantitative analyzes of protein abundance allowed the detection of differences in the proteome of control and exposed fish groups, and between male and female fish (gender specific responses). The altered proteins play functions in carbohydrate metabolism and energy production, ATP synthesis, cell structure maintenance, cellular transport, protein folding, stress response, detoxification and protease inhibition. These changes provide additional insights relative to the toxicity of anatoxin-a in fish. Taking into account the short time of response considered (5 min of response to the toxin), the changes in the proteome observed in this work are more likely to derive from fast occurring reactions in the cells. These could occur by protein activity regulation through degradation (proteolysis) and/or post-translational modifications, than from a differential regulation of gene expression, which may require more time for proteins to be synthesized and to produce changes at the proteomic level.

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1. Introduction

When a combination of environmental conditions (light, pH, temperature) occurs in freshwater ecosystems, usually associated with eutrophication, an outburst of cyanobacteria-a cyanobacterial bloom-can happen (Sindermann, 2005). Some species of cyanobacteria are known for having toxic strains (Valerio et al., 2010), which can produce toxic secondary metabolites (cyanotoxins). Outbreaks of these toxic cyanobacterial strains are known as harmful algal blooms (Cyano-HABs). Several studies suggested that the bioaccumulation of cyanotoxins such as microcystins, saxitoxins and also anatoxin-a, in aquatic biota can occur, increasing the risk of exposure to consumers (Ibelings and Havens, 2008). The naturally occurring neurotoxin anatoxin-a (2-acetyl-9-azabicyclo[4.2.1]non-2-ene) can be produced by several genera of freshwater cyanobacteria such as *Anabaena*, *Aphanizomenon*, *Arthrospira*, *Cylindrospermum*, *Microcystis*, *Nostoc*, *Oscillatoria*, *Phormidium*, *Planktothrix* and *Raphidiopsis* (Osswald et al., 2007a; Pelaez et al., 2010). Cyanobacterial strains and blooms producing anatoxin-a have occurred mainly in North America, Asia (North Korea and Japan) and Europe (Harada et al., 1993; Park et al., 1998, 1993). To our knowledge, the highest record concentration of

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anatoxin-a from a cyanobacterial bloom sample is 8000 μg g^{-1} dry weight that occurred in France (Gugger et al., 2005). Some occurrences of anatoxin-a have resulted in animal deaths, mainly dogs (Edwards et al., 1992; Hamill, 2001) but also cows (Stevens and Krieger, 1988) and flamingos (Krienitz et al., 2003).

Under natural conditions, the degradation of anatoxin-a is influenced by pH, light and the activity of microorganisms (Rapala et al., 1994), being converted into products with reduced toxicity (Stevens and Krieger, 1991). In conditions expected in an eutrophic lake bloom, this toxin's half-life was estimated to be about 24 h (Pawlik-Skowrońska and Toporowska, 2011). However, under laboratory conditions, this period may increase to five days (Pawlik-Skowrońska and Toporowska, 2011). Due to the instability of anatoxin-a, it is less common for animals to be exposed for long periods of time.

Anatoxin-a is a competitive agonist that can bind to the acetylcholine receptors at neuromuscular junctions affecting signal transmission between neurons and muscles, causing muscle cell overstimulation (Carmichael, 1994). This disturbance can affect respiratory muscles that consequently lead to disruption of oxygen supply to the brain. This results in convulsions and ultimately death by acute asphyxia (Dittmann and Wiegand, 2006). Other acute effects observed in vertebrates exposed to anatoxin-a include loss of coordination, decreased locomotor activity, muscle fasciculations, irregular breathing, tremors and altered gait (Carmichael et al., 1977; Rogers et al., 2005; Stolerman et al., 1992). In other experiments with carps and goldfish, behaviors such as rapid opercular movement, abnormal swimming (Osswald et al., 2007a) and muscle rigidity (Carmichael et al., 1975) were observed. Oberemm et al. (1999) described the alterations in heart rates in zebrafish embryos after an exposure to anatoxin-a.

This toxin has a rapid lethal effect (2–7 min), with an i.p. median lethal dose (LD_{50}) of pure toxin in mouse of 250 μg kg^{-1} (WHO, 2009). Since fish are vertebrates, the mechanism of action for anatoxin-a exposure should be similar to that of rodents. However other cellular processes such as biotransformation, which may contribute to a differential susceptibility among species and thus are necessary to understand, remain poorly described in the case of anatoxin-a (Osswald et al., 2011).

Exposure to cyanotoxins occurs mainly by dermal contact, ingestion of water or cyanobacterial scum (WHO, 2009). In humans, it is also possible to occur by intravenous exposure during hemodialysis when insufficiently purified water is available (Osswald et al., 2007b).

In the USA, the Washington State Department of Health recommended, as a provisional short-term recreational guideline, a value of 450 μg anatoxin-a L^{-1} (Pawlik-Skowrońska and Toporowska, 2011). However, the New Zealand Ministry of Health established 6 μg L^{-1} as the Provisional Maximum Acceptable Value for anatoxin-a in drinking-water (Wood et al., 2006). Such disparities should alert to the lack of information regarding this cyanotoxin.

Despite the negative effects of anatoxin-a, studies with this toxin have provided information about the properties of acetylcholine receptors in the nervous system and general acetylcholine-mediated neurotransmission functioning, becoming a helpful pharmacological probe (Parsons et al., 2000).

New evidences at molecular level may contribute to improve our current knowledge of the toxicity at species level. Moreover, it may help to identify novel anatoxin-a molecular targets. In this study we used the brain and muscle tissues of the fish model *Danio rerio* (zebrafish) and the 2DE method to characterize the differential protein expression in male and female zebrafish exposed to anatoxin-a, followed by identification of altered proteins by mass spectrometry.

This study aimed to provide a better understanding of the protein changes in the brain and muscle tissues of zebrafish after acute exposure to the toxin, and in this regard to offer additional information about the toxicity mechanisms and the effects of this neurotoxin in fish.

2. Materials and methods

2.1. Chemicals

The (±)-anatoxin-a fumarate used in this experiment was supplied by Tocris (Cat. No. 0789, U.K.). All other reagents used in this study were 98% purity grade or superior.

2.2. Animal maintenance

Adult one-year old zebrafish were retrieved from the Station of Maritime Zoology “Dr. Augusto Nobre” of the Faculty of Sciences of University of Porto (FCUP), where they were born. The animals (0.79 ± 0.27 g average weight) were distributed by 10 L tanks in a recirculating water system, thermo regulated at 28 ± 1 °C and with photoperiod set at 14 h light/10 h dark. Twenty fish were kept in each aquarium (2 fish L^{-1}), separated by gender, and fed twice a day with TetraMin food flakes (Tetra, Germany) and live *Artemia* nauplii, for two months before the exposure experiment.

2.3. Exposure experiment

Zebrafish were separated into female and male. For each gender two other groups were considered, the control and the anatoxin-a treatment group. For statistical purposes, we used three replicates for each experimental group. To prepare zebrafish for the i.p. injection we used the procedure described by Kinkel et al. (2010). The experiments were carried out in accordance with the guidelines for the care of laboratory animals and ethical guidelines for investigation in conscious animals set by the General Directorate of Veterinary of Portugal.

A stock solution of anatoxin-a fumarate (Tocris Bioscience, U.K.) in phosphate buffer saline (PBS) (pH 7.4) was prepared (0.59 μg of anatoxin-a μL^{-1} PBS). Injection solutions of desired concentrations of anatoxin-a (0.05–0.8 μg of anatoxin-a g^{-1} of fish body weight; bw) were freshly obtained for the preliminary and definitive assay by appropriate dilution of the stock solution with PBS.

In a preliminary assay, we administered, via i.p. injection, 3 μl per gram of bw of several doses of anatoxin-a in order to determine the lethal dose for zebrafish. Individuals died with the administration of 0.8 μg of anatoxin-a g^{-1} of fish bw within 10 min. This lethal dose of 0.8 μg anatoxin-a g^{-1} bw was selected for the definitive assay. Exposure of zebrafish to anatoxin-a was done by i.p. injection of an anatoxin-a solution of 0.8 μg anatoxin-a g^{-1} bw. The control fish received an i.p. injection of the PBS solution. Afterwards, fish were quickly transferred to a recipient with water at room temperature where they were kept for 5 min. Then, fish were rapidly euthanized by decapitation. A portion of the lateral muscle and the entire brain were collected, weighted and stored at −80 °C, until subsequent analysis. Brains and muscle tissues from 3 fish were dissected and pooled comprising respectively a single brain and muscle replicate sample. The sampling was repeated 3 times enabling, in total, 3 replicate samples (n=3) for each experimental condition. These were immediately stored at −80 °C for subsequent 2DE analysis.

2.4. Sample preparation

Tissues were homogenized with Tris–HCl buffer (30 mM, pH 8.8) plus protease inhibitors (Halt™ Protease inhibitor cocktail,

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