Aquatic Toxicology 156 (2014) 161–168

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Acute effects of cadmium on osmoregulation of the freshwater teleost *Prochilodus lineatus*: Enzymes activity and plasma ions

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ARTICLE INFO

Article history:
Received 30 April 2014
Received in revised form 21 August 2014
Accepted 23 August 2014
Available online 30 August 2014

Keywords:
Carbonic anhydrase
Hypocalcemia
Ca^{2+}-ATPase
Na^+/K^+-ATPase
H^+-ATPase

ABSTRACT

Cadmium (Cd) is a trace element that is very toxic to fish. It is commonly found in surface waters contaminated with industrial effluents. When dissolved in water, Cd can rapidly cause physiological changes in the gills and kidneys of freshwater fish. The objective of this study was to evaluate the acute effects of Cd on the osmoregulation of the Neotropical fish *Prochilodus lineatus*. Juvenile fish were exposed to Cd at two concentrations [1 (Cd1) and 10 (Cd10) $\mu g\,L^{-1}$] for 24 and 96 h. The effects of Cd were evaluated through the analysis of ions (Na^+, K^+, Ca^{2+}, and Cl^-) and plasma osmolality, and by measuring the activities of enzymes involved in osmoregulation obtained from the gills and kidney. Fish exposed to Cd for 24 and 96 h showed a decrease in Na^+/K^+-ATPase activity in the gills and kidney. The activity of carbonic anhydrase decreased in the gills after 24 h and in both tissues after 96 h of Cd exposure. The gill Ca^{2+}-ATPase activity also decreased with Cd exposure, with a concomitant drop in the plasma concentration of Ca^{2+}. Despite the hypocalcemia, there were no changes in the concentration of the ions Na^+, K^+, and Cl^- or in plasma osmolality. Among the enzymes involved in ion transport, H^+-ATPase was the only enzyme that showed increased activity in gills, whereas its activity in kidney remained unchanged. The results of this study demonstrate that waterborne Cd at the maximum concentrations set by Brazilian guidelines for freshwater affects the gills and kidney functions of *P. lineatus*. Acute exposure to Cd resulted in the decrease of the activity of enzymes, which culminated with the loss of the fish's ability to regulate the levels of calcium in the blood, leading to hypocalcemia.

1. Introduction

Metal contamination in freshwater ecosystems has increased in the past several decades, resulting in toxic effects to wildlife and damage to the environment. Cadmium (Cd) is a non-essential trace metal that is extremely toxic to aquatic biota. Surface waters normally contain relatively low concentrations of Cd; however, residues generated from industrial activities, mining, disposal of batteries, and use of fertilizers have contributed to the additional amounts of this contaminant in inland waters (Sorensen, 1991).

Fish are highly susceptible to Cd contamination (EPA, 2001). The toxic effects of Cd on fish are persistent and can be detected within a few hours of exposure (De La Torre et al., 2000). Chronic and sub-chronic exposure models have recently been used in ecotoxicology studies (Kamunde and MacPhail, 2011; Maunder et al., 2011; Cao et al., 2012); however, since the toxic effects of Cd are quickly established in fish, it is important to identify the physiological and biochemical alterations caused by acute exposure to this metal. Freshwater fish mainly absorb waterborne Cd through their gill epithelia; hence, gills are the first target organs of xenobiotics (Verbost et al., 1988). Once inside the organism, Cd enters the blood circulation to reach other organs and accumulates most significantly in kidney, followed by liver and gills (Pretto et al., 2011).

In freshwater fish, the gills and kidneys represent the main organs responsible for osmotic control and acid–base regulation (Gilmour and Perry, 2009). In the presence of Cd, modifications in enzymatic activities and in membrane transport systems are among the first changes that can be detected (Viarengo, 1989). Immediately after exposure to Cd, ionic regulation is compromised (McGeer et al., 2000), leading mainly to hypocalcemia, which is

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http://dx.doi.org/10.1016/j.aquatox.2014.08.009
0166-445X/

caused by the inhibition of the basolateral Ca^{2+}-ATPase in gill cell membranes (Verbost et al., 1988). In addition to the role of basolateral Ca^{2+}-ATPase (CATPase) in calcium absorption, Na^+/K^+-ATPase (NKA), H^+-ATPase (HATPase), and carbonic anhydrase (CA) present in the gills and kidney are also involved in the uptake of electrolytes from water and glomerular filtrate, respectively, and in the control of systemic pH by regulating the elimination of H^+ or HCO_3^- (Perry et al., 2003). The enzyme CA catalyzes the hydration of CO_2, generating HCO_3^- and H^+; these ions are involved in the transport of Na^+ and Cl^- (Boisen et al., 2003), which are also affected by the elimination of H^+ caused by the apical H^+-ATPase (Lin and Randall, 1991; Perry and Fryer, 1997). The activity of NKA in the basolateral membrane results in an electrochemical gradient that causes calcium to enter the cells through the apical side. Thus, the activities of these enzymes are essential for the uptake of ions and maintenance of ionic balance in freshwater fish (Lin and Randall, 1991; Perry et al., 2003; Evans et al., 2005).

There are considerable differences in Cd sensitivity among fish species and it is not possible to make definitive statements in relation to exposure concentrations that induce acute toxicity (McGeer et al., 2012). In Brazil, the emission of toxic substances, such as metals, to aquatic ecosystems is regulated by the Brazilian National Environmental Council (CONAMA, 2005). But the maximum acceptable values in the present national regulation are mainly based on international criteria like those from the United States Environmental Protection Agency (EPA) and the European Union (E.U.) regulations. Consequently, they do not consider the potential deleterious effects of pollutants to Brazilian native species (Martins and Bianchini, 2011). *Prochilodus lineatus* is a Neotropical fish species of great ecological significance that inhabits the largest South American river basins and is sensitive to metals, such as aluminum (Camargo et al., 2009), lead (Monteiro et al., 2011), and copper (Nascimento et al., 2012), nonetheless there is no information available regarding Cd effects on this fish species.

In this context, the objective of the present study was to evaluate the acute effects of waterborne Cd at the maximum concentrations set by Brazilian guidelines for freshwater on juveniles of *P. lineatus*. The parameters evaluated included the activity of the enzymes CATPase, HATPase, NKA, and CA in the gills and kidney, as well as the osmolality and plasma concentrations of Na^+, K^+, Ca^{2+}, and Cl^-, which would thus characterize the initial effects of Cd on the key components of osmoregulation in freshwater fish and the mechanism by which Cd affects plasma ion composition.

2. Materials and methods

2.1. Animals

Juvenile *P. lineatus* (12.4 ± 1.2 g; 11.2 ± 3 cm, $n = 251$) specimens were supplied by the Fish Culture Station of the State University of Londrina (EPUEL). Fish were acclimated for at least 5 days in tanks (300 L) containing non-chlorinated water, under constant aeration, and photoperiods of 12 h light and 12 h dark. During the acclimation period, fish were fed every 2 days with commercial fish feed (Guabi®, protein content of 36%). Feeding was suspended 24 h before and during the toxicity tests. The physical and chemical parameters of the water were monitored using a multi-parameter water quality meter (Horiba U-50).

2.2. Experimental protocol

Following acclimation, the animals were subjected to static acute toxicity tests for 24 and 96 h in 100-L glass tanks containing 80 L of water. Six to eight individuals were placed in each tank, keeping a maximum density of 1 g of fish per liter of water. Three groups were formed for each experimental period: a control (CTR) group, where fish were only exposed to non-chlorinated water, and two experimental groups, where fish were exposed to 1 $\mu g\,L^{-1}$ (Cd1) or 10 $\mu g\,L^{-1}$ (Cd10) of Cd, added from a 1 $mg\,L^{-1}$ cadmium chloride ($CdCl_2$) stock solution. All tests were carried out in duplicate. Cd concentrations were defined according to the maximal allowable concentrations of Cd in classes 1 and 2 (1 $\mu g\,L^{-1}$) and 3 and 4 (10 $\mu g\,L^{-1}$) freshwater, as defined by Brazilian legislation (CONAMA, 2005).

After the exposure period, the fish were anesthetized with benzocaine (0.12 $g\,L^{-1}$) and blood was drawn from the caudal vein into a pre-heparinized syringe. After blood collection, the animals were killed by medullar section. The gills and kidneys were removed using the procedure approved by the Animal Ethics Committee of the State University of Londrina (Process 35004.2011.18).

Blood samples were then transferred to 1.5-mL plastic tubes and kept on ice. Gill filaments were washed and separated from the gill arches, and together with kidney samples, were stored in plastic tubes containing the appropriate buffers for measurement of the activity of the enzymes NKA, HATPase, CATPase, and CA. All samples were kept frozen (−80 °C) until analysis.

The water temperature, pH, dissolved oxygen, and conductivity were monitored during the entire experiment. Water samples were collected after each experimental time, fixed with HNO_3 ($pH \leq 2$), and analyzed by electrothermal atomic absorption spectrometry, using an atomic absorption spectrometer equipped with a graphite furnace atomizer (Perkin Elmer A700), to determine Cd concentrations. Total Cd concentration was measured in unfiltered water samples, whereas the concentration of dissolved Cd was determined in filtered water samples (0.45-μm filter)

2.3. Plasma analyses

Blood samples were centrifuged (10 min; 1870 × *g*), and plasma samples were frozen (−20 °C). Osmolality was measured by determining the freezing point depression using an osmometer (Osmomat 030, Gonotec, Germany). A commercial test kit (Labtest Diagnóstica, Brazil) was used to determine the concentration of chloride by the mercury thiocyanate method using a microplate spectrophotometer (VictorTM, PerkinElmer) at 470 nm. The concentrations of sodium and potassium were determined in plasma samples diluted in deionized water (1:100) using a flame photometer (Analyser 900, Brazil). Calcium concentration was measured using the flame atomization method with an atomic absorption spectrometer (Perkin Elmer A700). Plasma samples were diluted to a ratio of 1:40 in 1% lanthanum chloride as a modifier.

2.4. Determination of NKA activity

NKA activity was determined in the homogenized fractions of gills and kidney according to the method described by Quabius et al. (1997). This method is based on the production of inorganic phosphorus in a medium after incubation in the presence and absence of ouabain. The excised organs were immersed in SEI buffer (0.3 M sucrose, 0.1 mM Na_2EDTA, 30 mM imidazole, 0.035% β-mercaptoethanol, pH 7.4) and stored in ultrafreezer at −80 °C. Samples were homogenized in the SEI buffer with Triton X-100 (1:1000, w/v), centrifuged (13,600 × *g*, 15 min, 4 °C), and the supernatant was used for the enzyme assay and for the determination of total protein (Lowry et al., 1951). Each sample was incubated with a buffer solution (100 mM NaCl, 8 mM $MgCl_2$, 30 mM imidazole, 0.1 mM EDTA, 3 mM ATP, pH 7.6) containing KCl (13 mM) or ouabain (2.5 mM). After a 30-min incubation in the dark, the reaction was stopped by adding a 1:1 mixture of 8.6% TCA and a color reagent (0.66 mM H_2SO_4 + 9.2 mM ammonium molybdate + 0.33 mM $Fe_2SO_4 \cdot 7H_2O$). A 650 mM phosphate solution