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Zinc causes acute impairment of glutathione metabolism followed by coordinated antioxidant defenses amplification in gills of brown mussels *Perna perna*

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ABSTRACT

Zinc demonstrates protective and antioxidant properties at physiological levels, although these characteristics are not attributed at moderate or high concentrations. Zinc toxicity has been related to a number of factors, including interference with antioxidant defenses. In particular, the inhibition of glutathione reductase (GR) has been suggested as a possible mechanism for acute zinc toxicity in bivalves. The present work investigates the biochemical effects of a non-lethal zinc concentration on antioxidant-related parameters in gills of brown mussels *Perna perna* exposed for 21 days to 2.6 μM zinc chloride. After 2 days of exposure, zinc caused impairment of the antioxidant system, decreasing GR activity and glutathione levels. An increase in antioxidant defenses became evident at 7 and 21 days of exposure, as an increase in superoxide dismutase and glutathione peroxidase activity along with restoration of glutathione levels and GR activity. After 7 and 21 days, an increase in cellular peroxides and lipid peroxidation end products were also detected, which are indicative of oxidative damage. Changes in GR activity contrasts with protein immunoblotting data, suggesting that zinc produces a long lasting inhibition of GR. Contrary to the general trend in antioxidants, levels of peroxiredoxin 6 decreased after 21 days of exposure. The data presented here support the hypothesis that zinc can impair thiol homeostasis, causes an increase in lipid peroxidation and inhibits GR, imposing a pro-oxidant status, which seems to trigger homeostatic mechanisms leading to a subsequent increase on antioxidant-related defenses.

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1. Introduction

The aquatic environment receives numerous anthropogenic compounds, such as polycyclic aromatic hydrocarbons, polychlorinated biphenyls, pesticides and metals (Martínez-Álvarez et al., 2005). Most of these substances are potent oxidants and may generate cellular imbalance and oxidative stress in aquatic organisms (Winston, 1991). Several ecotoxicological studies have been carried out to identify and characterize the modulation of antioxidant defenses and other biochemical systems, improving our knowledge about how pollutants can interact with living organisms. In this regard our study focuses on the potential mechanism for zinc toxicity in bivalves.

Zinc is a transition metal that participates in the modulation of regulatory proteins and cellular activities (Oteiza and Mackenzie, 2005). It also plays antioxidant roles: zinc deficiency is commonly related to increase in oxidants, cellular damage and modulation of antioxidant defenses (Oteiza, 2012), whereas zinc supplementation can counteract these effects in several malignancies and diseases (Chasapis et al., 2012), as well as act as an antidepressant (Brocardo et al., 2007). On the other hand, higher zinc concentrations can induce apoptosis and oxidative stress (Formigari et al., 2007), highlighting the dual role of this metal as oxidant/antioxidant and pro-apoptotic/anti-apoptotic agent.

Zinc is also an essential micronutrient abundant in invertebrates such as bivalves (Wong et al., 2000; Shi and Wang, 2004). In unpolluted areas, zinc is found in water at nanomolar levels, reaching micromolar values in metal-contaminated environments (García et al., 2008; Srinivasa Gowd and Govil, 2008; Voets et al., 2009). High levels of zinc in the sediment can also be correlated with levels of this metal in tissues of bivalves (Rebelo et al., 2003).

It has been proposed that soluble zinc and other metals are taken up mainly by gills and mantle of bivalves, while their particulate forms are taken up mainly by the digestive organs and stored in the digestive gland (George and Pirie, 1980; Wang, 2001; Cooper et al., 2010). The tolerance of these organisms to zinc exposure is similar to other invertebrates, with a LC_{50} of approximately 6.5 mgL^{-1} (~100 μM) for brown mussels *Perna perna* (Franco et al., 2006), 3.20 mgL^{-1} (~50 μM) for green mussels *Perna viridis* (Yap et al., 2004) and 16.4 mgL^{-1} (~250 μM) for the clam *Ruditapes philippinarum* (Dap et al., 1998). Bivalves also present an evident ability for zinc bioaccumulation (Chong

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and Wang, 2001), as part of a common mechanism of defense against exposure to metals. This occurs by bioaccumulation in metal-rich granules or scavenging by metallothionein-like proteins, generating a metabolically inactive metal pool. This mechanism of defense involves subcellular-partitioning processes, as well as regulation of metal uptake and efflux rates (Chen et al., 2011). An interesting study about the strategies associated with zinc tolerance in clams *Ruditapes decussatus* exposed to zinc 100μgL^{-1} (1.5 μM) during 40 days has previously found: 1) prominent bioaccumulation in gills and digestive gland; 2) storage as metal-rich granules; 3) zinc remobilization among cell compartments and 4) strong induction of metallothionein-like protein in gills (Serafim and Bebianno, 2007).

The toxicity of zinc to bivalves has been demonstrated at different levels, such as mortality in adults, embryos and gametes (Nadella et al., 2009; Fathallah et al., 2010), decrease in oxygen consumption and metabolic dysfunction (Devi, 1995), as well as modulation of the antioxidant system and induction of oxidative stress (Geret and Bebianno, 2004; Franco et al., 2006). For brown mussels *P. perna*, an acute exposure to a nonlethal dose of zinc chloride (10 μM) causes glutathione reductase (GR) inhibition along with an increase on the antioxidant defenses as a probable adaptive response, while, at higher doses, zinc induced oxidative damage and antioxidant disturbance. Our group has previously demonstrated that GR inhibition is a plausible mechanism for zinc toxicity in mussels, fish and mammals, affecting cellular glutathione metabolism (Franco et al., 2006, 2008a, 2008b). We have also demonstrated that the electrophilic compound 1-chloro-2,4-dinitrobenzene (CDNB) also decreases GR and thioredoxin reductase (TrxR) activity in Pacific oysters *Crassostrea gigas*, increasing the susceptibility of these animals to oxidative stress (Trevisan et al., 2012). These data suggest that inhibition of thiol-recycling systems such as glutathione/glutathione reductase and thioredoxin/thioredoxin reductase can be important toxicity factors for bivalves. Therefore, a time course experiment (2, 7 and 21 days) was devised in order to evaluate how GR and other antioxidant-related parameters are affected by short- and long-term zinc exposure in gills of brown mussels *P. perna*. At the beginning (2 days) GR inhibition was confirmed, along with disturbance of thiol homeostasis. This initial imbalance was followed by an increase in antioxidant defenses after 7 and 21 days, a possible adaptive response to cope with oxidative stress induced by zinc exposure.

2. Materials and methods

2.1. Chemicals

Zinc chloride, nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), disulfide glutathione (GSSG), reduced glutathione (GSH), cumene hydroperoxide, CDNB, glucose 6-phosphate, hydrogen peroxide (H_2O_2), xanthine oxidase, cytochrome c, perchloric acid (PCA), xylenol orange, thiobarbituric acid, bovine serum albumin, phenylmethylsulfonyl fluoride, protease inhibitor cocktail, 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) reagents and mouse monoclonal anti-β-actin HRP conjugated antibody were from Sigma-Aldrich (São Paulo, Brazil); nitrocellulose membranes, from Bio Rad (Berkeley, USA); rabbit anti-GR and anti-Prx6, and goat anti-rabbit IgG were from Abcam (Cambridge, USA); molecular-weight standards from GE Healthcare (Rio de Janeiro, Brazil); and γ-glutamyltranspeptidase (GGT) commercial kit from Biotecnica (Varginha, Brazil). All other reagents used were of analytical grade.

2.2. Animals and exposure conditions

Adult brown mussels *P. perna* (8–10 cm shell length) were obtained from a commercial aquaculture facility at Ribeirão da Ilha, Florianópolis (Brazil). The use of animals was performed in accordance with the policy of the Federal University of Santa Catarina. Animals were acclimated during 7 days under laboratory conditions (22–25 °C, 12 h light/dark) in plastic aquaria (1 L seawater/animal) and fed daily with a cultured *Chaetoceros muelleri* algae suspension.

In a previous study by our laboratory (Franco et al., 2006) 10 μM zinc chloride caused disturbance of the antioxidant system and oxidative damage after 2 days of exposure in brown mussels. It is a non-lethal concentration and corresponds at approximately 1/10 of $LC50_{(96h)}$, according to a preliminary test. Therefore, in order to investigate differential modulation of the antioxidant system by short and long-term exposure at lower concentrations, animals were exposed to 2.6 μM zinc chloride and killed after 2, 7 or 21 days. Non-exposed animals were used as control group.

A total of 8 aquaria were used, with 2 aquaria per exposure group and 6 animals per aquarium ($n = 12$ animals per group), according to the depiction in Fig. 1: At the beginning of the experiment (day 0), two aquaria (12 animals) were selected for zinc exposure for 21 days. At the 14th day of experiment, two additional aquaria were exposed to zinc for 7 days, and at the 19th day two extra aquaria were exposed to zinc for 2 days. The last 2 aquaria were maintained all the time with clean seawater. At the 21st day, all animals were sacrificed, gills were removed and stored at −80 °C for biochemical analysis, except for thiol analysis, which was performed at the same day as tissue collection. During the experimental period, the water was changed daily and supplemented with zinc chloride during the appropriate exposure period. Animals were fed daily with cultured *C. muelleri* algae suspension for 1 h prior to the water change to limit prolonged interaction between zinc and food. According to this exposure protocol, all animals were maintained under laboratory conditions during the same period (total of 21 days), diminishing biochemical and physiological alterations due to different acclimation or sampling day error.

2.3. Metal analysis

Zinc was determined in aliquots of seawater at the first day of exposure for each group and performed in a graphite furnace atomic absorption spectrometer with a Zeeman background correction PerkinElmer AAnalyst-600, after convenient dilution. Certified reference materials SLRS-4 and NASS-5 (National Research Council Canada) were used to verify the analytical accuracy. Zinc content in the seawater (0.16 ± 0.08 μM) was increased to 2.59 ± 0.9 μM ($N = 3$ with 3 replicates per sample). During the following sections of the text we will refer simply as 2.6 μM of zinc.

2.4. Glutathione, non-protein and protein thiols

Gills were immediately removed and approximately 100 mg of fresh tissue of individual animals were deproteinized in 900 μL of 0.5 M perchloric acid (PCA) and centrifuged at 15,000 *g* for 2 min at 4 °C. The acid supernatant was used to measure total glutathione (as the sum of reduced and disulfide form of glutathione; GSH-t) and non-protein thiols (such as glutathione and cysteine; NPSH), while the pellet was used to measure protein thiols (PSH). The NPSH and PSH levels were determined by a colorimetric method (Ellman, 1959), while GSH-t was determined by an enzyme-coupled assay (Akerboom and Sies, 1981).

2.5. Enzymatic analysis and oxidative markers

Portion of gills (approximately 400 mg, from the same individuals used for thiol analysis) were homogenized in 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer pH 7.0 (1:4, weight:volume) and centrifuged at 5,000 *g* for 5 min at 4 °C. Pellets (nuclei and cellular debris) were discarded, and the supernatant was further centrifuged at 20,000 *g* for 30 min at 4 °C. The supernatant was used for peroxide and enzymatic measurements, while the pellet (membrane vesicles, mitochondria, lysosomes) was used for the

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