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Antioxidant responses in different body regions of the polychaeta *Laeonereis acuta* (Nereididae) exposed to copper

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ABSTRACT

Antioxidant enzymes, total antioxidant capacity (TOSC) and concentration of reactive oxygen species (ROS) were measured in anterior (A), middle (M) and posterior (P) body regions of *Laeonereis acuta* after copper (Cu; 62.5 μg/l) exposure. A catalase (CAT) activity gradient observed in control group (lowest in A, highest in P) was not observed in Cu exposed group. Glutathione-*S*-transferase (GST) activity in A region of Cu group was higher than in A region of the control group. DNA damage (comet assay) was augmented in the A region of Cu group. Since copper accumulation was similar in the different body regions, sensitivity to copper in A regions seems to be related to lowest CAT activity. In sum, copper exposure lowered TOSC, a result that at least in part can be related to lowering of antioxidant enzymes like CAT. DNA damage was induced in the anterior region, where a lower CAT activity was observed.

1. Introduction

The nereidid polychaeta *Laeonereis acuta* is an epifaunal animal broadly distributed in shallow waters of estuaries, and previous studies have characterized this organism as a selective deposit feeder (Bemvenuti, 1998). The Nereididae family is described as anoxic and hypoxic tolerant, and several responses of the antioxidant defense system (ADS) have been described in this group (Abele-Oeschger et al., 1994; Abele-Oeschger and Oeschger, 1995; Abele et al., 1998; Rosa et al., 2005).

Copper pollution in the aquatic environment results from natural and anthropogenic sources such as mine washing or agricultural leaching. Although copper is a trace element essential to life it is also one of the most toxic heavy metals (Tóth et al., 1996). One of the main toxic mechanisms of this metal is due to oxidative stress generation through Fenton and Haber–Weiss reaction (Furuno et al., 1996). Pourahmad and O'Brien (2000) showed that ROS formation, glutathione oxidation and lipid peroxidation were induced in hepatocytes exposed to copper. Lloyd and Phillips (1999) observed the formation of double-strand breaks, 8-hydroxydeoxyguanosine and putative intra-strand cross-links mediated by copper, iron and nickel in salmon sperm DNA. In the freshwater olygochaeta *Tubifex tubifex*, higher concentrations of copper and lead were revealed in its posterior region than in its anterior region when the organisms were collected in a polluted site (Lucan-Bouché et al., 1999). In another study, the same species was exposed to different copper concentrations, and higher catalase (CAT) activity and reduced glutathione-*S*-transferase (GST) activity were observed (Mosleh et al., 2006). Morgan et al. (1989) verified that *Dendrodrilus rubidus* and *Lumbricus rubellus* sampled at a soil contaminated with cadmium showed major accumulation of this metal within the posterior alimentary canal.

The estuarine worm *L. acuta* (Nereididae) was formerly used as a biological model in copper toxicity assays and environment monitoring studies (Geracitano et al., 2002, 2004a, b), mainly taking into account its antioxidants responses. Furthermore, a gradient of antioxidant enzymes activity along the body of this worm was verified (Rosa et al., 2005), exhibiting higher CAT and superoxide dismutase (SOD) activities in the posterior region and higher GST activity in the anterior region. This type of gradient also has been demonstrated in the capitellid worm *Heteromastus filiformis*, with a correlation between an external gradient of PO_2 and pH and an internal gradient of ADS (Abele et al., 1998). Taking into consideration the existence of an antioxidant gradient along the body of *L. acuta* and also the previous reports showing differential metal accumulation (copper and lead) in different

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body regions of annelids (Lucan-Bouché et al., 1999), the aim of this study was to analyze copper accumulation and antioxidant and oxidative damage responses in anterior, middle and posterior body regions of *L. acuta* after experimental exposure to copper.

2. Material and methods

2.1. Collected animals and maintenance

Specimens of the polychaeta *L. acuta* weighting between 60 and 120 mg were collected in a salt marsh in Saco do Justino of Patos Lagoon (32°05′S–052°12′W; RS, Brazil) during the winters of the years 2003, 2004 and in the 3 years 2005–7. The worms were transferred to the laboratory in an ice-cold container in order to reduce their metabolism. Once in laboratory, worms were maintained under 12:12 h light:dark cycle, saline water 10‰, pH 8.0 and 20 °C for all assays. For the chronic assays the protocol of acclimation (10 days) depicted by Geracitano et al. (2002) was followed. For the *in vitro* assays the acclimation was of 2 days without feeding, in order to clean the intestines of the employed organisms.

2.2. Chronic assays

In the chronic assays (14 days) the worms were divided into two experimental groups: one was the control group (Ctr) and the other group was exposed to nominal copper concentration of 62.5 μg/l ($CuSO_4 \cdot H_2O$ from Merck, Rio de Janeiro, Brazil). This concentration was selected taking into account that one of the more sensitive parameters to copper exposure in oligochaetas is cocoon formation, which is inhibited at concentrations of copper about 50–60 μg/l (International Programme on Chemical Safety, 1993). Worms were placed in glass dishes (6.0 cm diameter), filled with 100 ml of saline water (with or without copper). Animals in the chronic assays were fed *ad libitum* with frozen *Artemia salina* every 2 days and the water was then 100% renewed.

After the end of the exposure, the organisms were sacrificed, dissected and subdivided in three regions: anterior region (A, first 20 settiger segments), middle region (M, next 20 settiger segments) and posterior region (P, the rest of the body) (Rosa et al., 2005). Pools of A, M or P sections were formed to make one sample. Because of low tissue quantity in A region, few samples were analyzed in this region ($n = 3$–6). In the other body regions, number of samples analyzed varied between 5 and 10. The number of worms employed for each treatment varied between 30 (control) and 40 (copper).

For copper analysis samples of each body region were dried at 60 °C until constant weight. Total content of copper in each region was evaluated using an atomic absorption spectrometer with a graphite furnace (ZEEnit 60). One milliliter of nitric acid 65% was added to samples weighing more than 0.03 g (0.5 ml of nitric acid was added to samples weighing less than 0.03 g). After 24 h in this solution, samples were heated (60 °C) for 2 h. NIST 2976 was the certified material used for method validation.

For the enzymatic assays, pools of each region were homogenized (1:3 w/v) in ice-cold buffer with pH adjusted to 7.60 (20 mM Tris-base, 1 mM EDTA, 1 mM DL-dithiothreitol, 500 mM sucrose and 150 mM KCl). Homogenates were centrifuged at 9000*g* for 45 min (4 °C) and the supernatants were collected and stored at −80 °C and employed later to determine total protein content, and CAT and GST activities (Geracitano et al., 2002). All the determination assays were performed at least in duplicate. The total protein content in the supernatant of homogenate extracts was determined at 550 nm using a commercial diagnostic kit (Doles reagents, Brazil) based on the Biuret method. Number of samples varied between 3 and 6 for each treatment and body region. As previously stated, few samples from anterior body region were analyzed in virtue of tissue availability.

The activity of the enzyme CAT was quantified by the consumption of 10 mM of H_2O_2 at 240 nm according to Beutler (1975). The activity of the enzyme was expressed in CAT units/mg of proteins, where one unit (U) is the amount of enzyme that hydrolyzes 1 μmol of H_2O_2 per minute and per mg of proteins at 30 °C and pH 8.0. The activity of the enzyme GST was measured by following the conjugation of 1 mM glutathione and 1 mM 1-chloro-2,4-dinitrobenzene at 340 nm as described by Habig et al. (1974) and Habig and Jakoby (1981). The activity of the enzyme was expressed in GST units/mg of proteins, where one unit (U) is the amount of enzyme that conjugates 1 μmol of CDNB per minute and per milligram of protein at 25 °C and pH 7.0.

For total oxyradical scavenging capacity (total antioxidant capacity (TOSC) assay) pools of each body region from sampled worms were homogenized (1:4, w/v) in ice-cold phosphate buffer 50 mM, pH 7.50, plus NaCl (2.5%, w/v). Homogenates were centrifuged at 13,500*g* for 25 min (4 °C) and aliquots from the supernatant were collected for a second centrifugation at 33,000*g* during 60 min (4 °C) (Geracitano et al., 2004a). The supernatant of this last centrifugation was stored at −80 °C for TOSC measurements. Additional assays were conducted with the diet given to worms, *Artemia salina*, in order to analyze the antioxidant influence of the diet offered to the worms. Frozen *A. salina* was homogenized exactly as worm samples, and their antioxidant capacity determined. The total protein content in the supernatant was measured in triplicate as described above. TOSC was determined according to Winston et al. (1998) and Regoli and Winston (1999), using alpha-keto-γ-methiolbutyric acid (KMBA; 0.2 mM; Sigma, USA) as substrate. KMBA reacts with reactive oxygen species (ROS) producing ethylene. TOSC values reflect the sample ability to inhibit ethylene formation, through ROS scavenging. Two different kinds of ROS were generated *in vitro*. Peroxyl radicals were produced by thermal (35 °C) decomposition of 2,2′-azobis 2 methylpropionamidine dihydrochloride (ABAP; 20 mM; Aldrich) dissolved in potassium phosphate buffer 100 mM, pH 7.4. Hydroxyl radicals were produced using a Fenton system containing Fe^{3+} (2.7 μM)/EDTA (5.4 μM), and ascorbic acid (270 μM). Ethylene gas produced was measured using a Shimadzu GC-17A gas chromatograph equipped with an Agilent Technologies (USA) DB-5 capillary column (30 m × 0.25 mm × 0.25 μm) and a flame ionization detector (FID). TOSC values were calculated according to Winston et al. (1998) and referred to the total protein content in the homogenates. Number of samples analyzed varied between 6 and 10, for each body region and treatment.

The xylenol orange assay for lipid hydroperoxides (LPO) was performed as described by Monserrat et al. (2003). Frozen tissues of each region were homogenized in methanol (1:9 w/v) and centrifuged at 1000*g* for 5 min. LPO were determined using $FeSO_4$ (1 mM), H_2SO_4 (0.25 M) and xylenol orange (1 mM) added in this order. The assay mixture was measured at 580 nm in a microplate reader after 1 h of incubation at room tempeture. LPO values were expressed in terms of cumene hydroperoxide (CHP) equivalents, used as standard (1.75 nmol/ml).

The comet assay was executed according to Singh et al. (1988) and Steinert et al. (1998), with some modifications. Microscope slides were fully frosted and covered with 1% normal melting point agarose diluted in a solution with 0.04 Tris–acetate and 1 mM EDTA, cleaning the rear side with tissue and then drying. Each region (A, M and P, $n = 5$) of the two experimental groups was immediately homogenized in Petri dishes at the end of the 14-day experiment. The homogenates were filtered (pore: 145 μM) for cell separation. The cellular preparation was diluted (1:25 v/v) in Kenny's salt solution (400 mM NaCl, 9 mM KCl, 0.7 mM K_2HPO_4, 2 mM $NAHCO_3$, pH 7.50). After a second dilution (1:7.5 v/v) in 0.65% low-melting point agarose (diluted in Kenny's salt solution) the cellular preparations were added to the prepared frosted slide and roofed with a cover slip. Then, the slides were submitted to lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris–HCl, 10% dimethyl sulfoxide, 1% Triton X-100, and 1% sodium sarcosyl) and kept at 4 °C overnight. For unwinding DNA strands, slides were transferred into chambers filled with electrophoresis and unwinding buffer (10 M NaOH, and 200 mM EDTA, pH 13.00) for 15 min. Eelectrophoresis was carried out for 20 min at 25 V and 280 mA. Slides were washed with 400 mM Tris (pH 7.50) and stained with 80 μl of ethidium bromide. DNA migration was visually determined in 100 cells per slide randomly selected in an epifluorescence Zeiss-Axioplan microscope (400 magnification). Comets were classified into five different groups: 0 for intact nucleoids; 1, 2 and 3 for intermediary damage, and 4 for maximum damage. Results were expressed as scores, 0 represents absence of damage and 400 indicates the highest damage registered in the 100 nucleoids analyzed.. Four or five slides of each body region and treatment was analyzed.

DNA–protein cross-link (DNAPC) levels were determined using K^+/SDS precipitation assay, following the method of Costa et al. (1996). Worm tissues were lysed in 500 μl of 0.5% SDS, 1 mM PMSF, 20 mM Tris–HCl, pH 7.5. The DNAPC fraction was digested with 200 μg of proteinase K (15 units/mg protein) for 3 h at 50 °C. The samples were dyed using SyberGold (Fluka) fluorescent dye. The amount of DNA in the samples was determined using the fluorometer (Victor 2, Perkin-Elmer), with excitation and emission wavelengths 485 and 535 nm, respectively.

2.3. In vitro assays

In the first *in vitro* assay the worms were divided into three experimental groups: control group (Ctra); 250 μg of copper/l (C1a), and 1.6 mg of copper/l (C2a). Copper concentrations were selected taking into account copper accumulation observed in the chronic assay (C2a) and a concentration previously assayed (C1a) in an acute experiment by Geracitano et al. (2002). In the second *in vitro* assay the worms were divided into three experimental groups: control group (Ctrb), 10 μM of H_2O_2 (C1b), and 50 μM of H_2O_2 (C2b), taking into account the concentrations assayed by Rosa et al. (2005) in *in vivo* experiments aimed to evaluate hydrogen peroxide effects on different body regions of *L. acuta*. In both assays the organisms were also dissected and subdivided in different regions A, M and P as in the chronic assays.

Measurements of ROS were conducted to according to Ferreira-Cravo et al. (2007). Homogenates were obtained from the regions A, M and P of each experimental group of worms (1:4 w/v) in ice-cold buffer (320 mM sucrose, 20 mM HEPES, 1 mM $MgCl_2$, pH 7.40). Homogenates were centrifuged at 20,000*g* for 20 min (4 °C). The supernatants were obtained (166 μg of total proteins) and incubated at 25 °C during 30 min with 30 mM HEPES (pH 7.2), 200 mM KCl, 1 mM $MgCl_2$ and 16 μM of 2′,7′-dichlorofluorescin diacetate (H_2DCF-DA; Molecular Probes). The acetates groups of H_2DCF-DA are cleaved by intracellular esterases. After that, the non-fluorescent compound H_2DCF is oxidized by ROS to the fluorescent compound, DCF. The fluorescence intensity was determined, using a