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Metallothionein is up-regulated in molluscan responses to cadmium, but not aluminum, exposure

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Abstract Metallothionein (MTs) is a family of low molecular weight peptides with high cysteine content. In aquatic invertebrates, MTs play an important role in the detoxification of metals and they are often cited as useful biomarkers for toxic metal stress. The change in concentrations of metallothionein in response to aluminum and cadmium exposure was investigated in the soft tissues of two freshwater molluscs: *Lymnaea stagnalis* and *Dreissena polymorpha* that have contrasting feeding behavior and therefore metal accumulation profiles. The majority of added Al had precipitated in the bottom of tanks within 24 h, with an average of only 15.4% and 26.2% remaining in the water column of tanks containing snails and mussels, respectively. In contrast, most of the Cd did not precipitate but remained in solution (81.1% and 82.7%, respectively) between water changes. Both metals accumulated in the soft tissues of both animals, but only cadmium exposure led to an increase in metallothionein concentrations in the soft tissues. However, no significant difference was observed in the level of total protein in both tested molluscs upon exposure to Al and Cd compared to the corresponding control. Therefore, care should be taken in the use of metallothioneins as biomarkers for metal stress, since not all metallic stressors induce their expression.

Introduction

Metallothioneins (MTs) and metallothionein-like proteins (MTLPs) are a family of low molecular weight peptides with high cysteine content. In aquatic invertebrates, MTs play an important role in many physiological processes including homeostasis, protection against heavy metals and oxidant damage, metabolic regulation, sequestration and/or redox control (Mao et al., 2012). Whilst the roles of MTs and MTLPs may vary, numerous studies have implicated them in the detoxification of metals in aquatic invertebrates (Amiard et al., 2006) and they were often cited as useful biomarkers for toxic metal stress (Itziou and Dimitriadis, 2011; Ladhar-Chaabouni et al., 2012).

MTs readily associate with metal ions such as Hg^{2+} and Cd^{2+} and others with low charge states, owing to the presence of up to 18 soft-base thiol groups per molecule. They are not expected to associate as readily with 'hard' metal ions such as Al^{3+} which have high charge states and are therefore more likely to associate with hard (Lewis) bases such as phosphate and silicate. Whilst aluminum did not associate with MTs isolated from rat

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tissue (Vandervoet et al., 1992), there is some evidence of an association in the marine mussel, *Mytilus galloprovincialis* (Santiago-Rivas et al., 2007). Similarly, in the freshwater snail *Lymnaea stagnalis*, Al associates with heat stable proteins (the group to which MTs belong) to a greater extent than another hard metal Gallium (Walton et al., 2010). There is, however, no published evidence showing that Al itself increases the levels of expression of MTs or MTLPs in aquatic invertebrates.

The aim of this study was to investigate a possible role for MT or MTLPs in the detoxification of Al in freshwater invertebrates. To do this, we examined whether concentrations of MT in tissues of two freshwater molluscs, *L. stagnalis* and *Dreissena polymorpha*, increased in response to Al exposure. Cadmium (Cd) was used as a positive control as it is considered an efficient inducer of MT expression in all classes of aquatic invertebrate (Amiard et al., 2006; Strogyloudi et al., 2012).

Materials and methods

Experimental design

Mature *L. stagnalis* (3.0–3.5 cm shell length), and mature *D. polymorpha* (2.7–3.0 cm shell length) were collected from field sites in Cheshire and Greater Manchester, UK and acclimatized to aerated simulated, defined pond water (SDPW: 222 mg l^{-1} $CaCl_2$, 9.6 mg l^{-1} $MgSO_4$, 4 mg l^{-1} $KHCO_3$, 5.1 mg l^{-1} KNO_3, 58 mg l^{-1} $NaHCO_3$, pH 7.3) at 12 °C and on a 12:12 h light:dark regime for 10 days prior to the start of exposure. Snails or mussels were maintained in 10 l tanks (50 individuals per tank) and exposed for up to 20 days to either 500 mg l^{-1} Al [as $Al(NO_3)_3$] or 50 mg l^{-1} Cd [as $CdCl_2{\cdot}(5/2)H_2O$] in SDPW. Tanks containing no added metals acted as controls for both experimental species. The SDPW in all tanks was renewed every second day and re-dosed with metal. All tanks were continuously aerated. Snails were fed on lettuce *ad libitum* and mussels were fed on Interpet Liquifry Marine (Swell, UK). All solutions were prepared using ultrahigh purity (UHP) water and high-purity chemicals (Merck or Aldrich). Polypropylene plasticware was acid-washed and rinsed before use.

Metals analysis

Water samples were taken 1, 24 and 48 h after each water change and acidified for subsequent metal analysis. Six animals from each treatment were sampled on days 0, 10 and 20 to determine tissue accumulation of metals. The digestive gland (DG – the detoxificatory organ in molluscs – Simkiss, 1977) of individual snails and the whole soft tissues (WST) of individual mussels were oven-dried, weighed and digested in 2 ml of 30 wt.%/vol H_2O_2 and 2 ml ultrapure HNO_3, followed by dilution with 12 ml of UHP water. Samples were analyzed for Al and Cd (together with digest blanks for control) by inductively coupled plasma optical emission spectroscopy (ICP-OES) using a Perkin-Elmer Optima 5300. Detection limits were calculated as 3× the highest standard deviation value from a set of measurements.

Protein and metallothionein assay

Total protein and metallothionein concentrations in the whole soft tissues of mussels and the DG of snails were quantified. Tissues were weighed and homogenized in three volumes of the homogenization buffer (0.5 M sucrose; 20 mM Tris–HCl buffer, pH 8.6; 0.01% β-mercaptoethanol) using a motor driven glass homogenizer. The homogenates were centrifuged at 30,000*g* (Sorval RC 28S centrifuge) for 20 min. The supernatants were removed, one-half stored at −20 °C for later protein quantification, and the rest assayed for metallothionein using the method described by Linde and Garcia-Vazquez (2006). Cold (1.05 ml, −20 °C) absolute ethanol and 80 ml of chloroform were added for each 1 ml of the supernatant. Samples were mixed and centrifuged at 6000*g* for 10 min at 4 °C. Three volumes of cold ethanol were added to the supernatant and incubated at −20 °C for 1 h. These samples were centrifuged at 6000*g* for 10 min. The resulting pellets were washed with ethanol:chloroform:homogenization buffer (87:1:12) and centrifuged at 6000*g* for 10 min and then dried under a N_2 gas stream. The pellet was resuspended in 300 µl of 5 mM Tris–HCl, 1 mM EDTA, pH 7, and then added to 4.2 ml of 0.43 mM 5,5-dithiobis (nitrobenzoic acid) in 0.2 M phosphate buffer, pH 8, and left for 30 min at room temperature. The concentration of reduced sulfhydryl was measured by reading the absorbance at 412 nm, and comparing it to a standard curve with glutathione (GSH) as a standard reference. GSH contains one cysteine per molecule; the amount of metallothionein in the samples was estimated using the GSH standard, assuming that 1 mol of MT contains 18 mol of cysteine, as it does in Crustacea (Binz and Kägi, 1999).

Total protein in the supernatants was measured using Bradford's reagent (Sigma, UK; see also Bradford, 1976) using bovine serum albumin as the standard; A^{595} absorbances were measured 5 min after the addition of the Bradford reagent and concentrations calculated from the standard curve.

Statistical analyses

Data were analyzed using Microsoft Excel and SPSS (Version 14), using a Mann–Whitney *U* test with significance defined as either $p < 0.05$ or $p < 0.01$. Multiple comparisons were carried out using a Kruskall–Wallis test, followed by Mann–Whitney *U* with Hochberg's modified Bonferroni correction procedure to adjust for multiple comparisons. In these cases nominal alpha was set to 0.01.

Results and discussion

Metal concentrations in water are summarized in Table 1. The majority of Al added to the SDPW had precipitated within 24 h, with an average of only 15.4% and 26.2% remaining in the water column of tanks containing snails and mussels, respectively. Under neutral conditions in which most of the Al is predicted to be in its insoluble (colloidal) form, Al was significantly accumulated by the grazing freshwater snail, *L. stagnalis* (Desouky et al., 2003), and the filter-feeding bivalve *D. polymorpha* (Marie et al., 2006) used in this study, as well as the bivalve *Anodonta cygnea* (Kadar et al., 2001). In contrast, most of the Cd did not precipitate but remained in solution (81.1% and 82.7%, respectively) between water changes (48 h).

The two freshwater molluscan species: the snail *L. stagnalis* and the zebra mussel *D. polymorpha* used in this study represent organisms with contrasting feeding, and therefore metal