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Time course of lead induced proteomic changes in gill of the Antarctic limpet *Nacella Concinna* (Gastropoda: Patellidae)

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ABSTRACT

The effect of increasing levels of metals from anthropogenic sources on Antarctic invertebrates is poorly understood. Here we exposed limpets (*Nacella concinna*) to 0, 0.12 and 0.25 μg L^{-1} lead for 12, 24, 48 and 168 h. We subsequently quantified the changes in protein abundance from gill, using 2D gel electrophoresis and mass spectrometry. We identified several antioxidant proteins, including the metal binding *Mn*-superoxide dismutase and ferritin, increasing abundances early on. Chaperones involved in the redox-dependent maturation of proteins in the endoplasmic reticulum (ER) showed higher abundance with lead at 48 h. Lead also increased the abundance of *Zn*-binding carbonic anhydrase at 12 h, suggesting a challenge to acid-base balance. Metabolic proteins increased abundance at 168 h, suggesting a greater ATP demand during prolonged exposure. Changes in abundance of the small G-protein cdc42, a signaling protein modifying cytoskeleton, increased early and subsequently reversed during prolonged exposure, possibly leading to the modification of thick filament structure and function. We hypothesize that the replacement of metals initially affected antioxidant proteins and increased the production of reactive oxygen species. This disrupted the redox-sensitive maturation of proteins in the ER and caused increased ATP demand later on, accompanied by changes in cytoskeleton.

Significance: Proteomic analysis of gill tissue in Antarctic limpets exposed to different concentrations of lead (Pb) over a 168 h time period showed that proteomic changes vary with time. These changes included an increase in the demand of scavenging reactive oxygen species, acid-base balance and a challenge to protein homeostasis in the endoplasmic reticulum early on and subsequently an increase in energy metabolism, cellular signaling, and cytoskeletal modifications. Based on this time course, we hypothesize that the main mode of action of lead is a replacement of metal-cofactors of key enzymes involved in the scavenging of reactive oxygen species and the regulation of acid-base balance.

1. Introduction

The Antarctic environment is currently undergoing wide-ranging changes due to anthropogenic climate change and pollution [1]. One particular challenge is the transfer of metals from ice to the sea through increased melting and human pollution, affecting a number of Antarctic marine invertebrates, and in particular the limpet *Nacella concinna* [2].

Because of its toxicity to aquatic organisms and their potential to bioaccumulate, the effect of metals has been studied for several Antarctic marine species [3–8]. The relatively high concentrations of the metals Al, Co, Ni, Cu, Zn, Cd, Pb, Mn, Fe, As and Se in Antarctic waters have been attributed to water input from progressive deglaciation, soil composition, weathering of volcanic lithogenic rock and atmospheric deposition [9–13]. In addition, the natural background levels of trace metals have been elevated since the establishment of bases on the Antarctic continent [14–17]. During the austral summer the circumpolar vortex disappears and long-term records of mineral dust, black carbon and Pb in the South Pole and several coastal Antarctic stations indicate a greater transport of air masses poleward [18]. As a consequence, contamination of Antarctic snow by aerosols containing Pb has been widely documented and has generally been attributed to leaded gasoline [19], mining of non-ferrous metals, smelting or other anthropogenic sources in South America, Africa and Australia. Moreover, while the deposition of Pb in Antarctic snow has been decreasing in recent decades [20], metal emissions, including Pb, from human activities in Antarctica have the potential to cause environmental contamination in localized areas. For example, the main anthropogenic emissions within Antarctica are related to the burning of fuel for the operation of power plants, motorcycles, trucks, ships and aircrafts [21].

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Indeed, the increased levels of Pb concentrations recorded in the proximity of the research stations indicated an anthropogenic origin. [13]. For example, a recent metal analysis of tissues from the Antarctic clam (*Laternula elliptica*) showed high Pb levels nearby an Antarctic research station [17]. In previous studies, Antarctic seawater showed elevated concentrations of lead in values between 0.05 and 0.06 μg L^{-1}, including places near the meltwater input source [10,22,23].

Evolutionary adaptations to the Antarctic environmental conditions can influence the sensitivity of organisms to stress [24–27]. Most biological processes in the benthos and tidal zone of the polar seas, including growth, reproduction and metabolism are slow, which reduces the ability to detoxify and remove contaminants, making Antarctic organisms especially sensitive to additional environmental stress [28,29]. In combination with the projections of increasing basal melt rates in the Weddell Sea sector of the West Antarctic ice sheet [30], the sensitivity to pollutants has the potential to be particularly stressful in the near future. Specifically, dissolved metals can be absorbed from the water column directly into the gills of mollusks [2]. Furthermore, the bioaccumulation of contaminants, resulting in the increase in tissue concentration, can cause harmful effects in aquatic invertebrates, even when concentrations in water are too low to carry the danger of acute toxicity [31].

Antarctic limpets have been widely used to study physiological responses to environmental stress [32–39]. Previous studies conducted in Antarctica, including King George Island, showed that *N. concinna* accumulated a considerable amount of metals in different tissues [9,10,40,41]. Importantly, environmental stress, including exposure to different metals, led to the activation of the antioxidant system, an important indicator for the perturbation of the general redox system of the cell [2].

Proteomic analyses of the response to metals and other pollutants have been particularly successful in identifying novel patterns of cellular responses in temperate organisms [42,43], characterizing unique changes in protein abundance in response to specific stressors [43–48], including limpets [49,50]. Overall, the application of proteomics to study the response of Antarctic organisms to metal exposure can potentially provide insights into the effects of pollutants on the cellular responses in organisms with a slow metabolism and lower detoxification rate [51]. This is a promising approach, especially since proteomic studies on Antarctic organisms are largely absent [52,53]. Our results from exposure of Antarctic limpets to Pb detected changes in several cellular processes associated with scavenging of reactive oxygen species, acid-base balance, protein homeostasis, energy metabolism, cellular signaling, and cytoskeletal modification, which offer insights into why cold-adapted organisms may be particularly sensitive to pollutant stress.

2. Materials and methods

2.1. Animal collection and maintenance

The Antarctic limpets (>30 mm) were collected manually on rock surfaces at low tide during the austral summer 2010/2011 from King George Island, South Shetland Islands, Admiralty Bay at Plaza Point (62°04′14, 5″S; 58°24′11, 9″W) and Botany Point (62°06′15, 7″S; 58°21′14, 0″W). Limpets were acclimated to laboratory conditions at the Brazilian Antarctic Station Comandante Ferraz for 72 h in an air-conditioned laboratory. Limpets were placed in recirculating aquaria. Salinity was 35 psu, water temperature 0 °C and photoperiod 12/12 h. Genders were not distinguished. No mortalities occurred during the acclimation period.

2.2. Experimental design

Groups of limpets were exposed to lead dissolved in water at concentrations of 0.0 (control), 0.12 and 0.25 μg L^{-1}. The concentrations are, approximately, 2.5 and 5 times higher than those already observed in this particular environment. The exposure times were 12, 24, 48 and 168 h ($N = 6$ for each time point and treatment). Lead was added to the aquaria at the start of the experiments from a stock solution. We also tested the control group for lead concentration. Gill tissue was sampled from all treatment groups and subsequently kept at −80 °C. We choose to investigate gill tissue, as it is directly exposed to contaminated seawater, a metabolically highly active tissue, which provides direct comparisons with other studies on the effects of metals on cellular functions.

2.3. Homogenization

Frozen gill tissue was homogenized with an ice-cold ground-glass homogenizer in a ratio of 1:4 of tissue to homogenization buffer: 7 M urea, 2 M thiourea, 1% ASB-14 (amidosulfobetaine-14), 40 mM Tris-base, 0.5% immobilized pH 4–7 gradient (IPG) buffer (GE Healthcare, Piscataway, NJ, USA) and 40 mM dithiothreitol. The homogenate was subsequently centrifuged at room temperature for 30 min at 16,100 *g* and the supernatant was used for further processing. Proteins of the supernatant were precipitated by adding four volumes of ice-cold 10% trichloroacetic acid in acetone and incubated at −20 °C overnight. After centrifugation at 4 °C for 15 min at 18,000 *g*, the supernatant was discarded and the remaining pellet was washed with ice-cold acetone, and centrifuged again before being re-suspended through vortexing in rehydration buffer: 7 M urea, 2 M thiourea, 2% CHAPS (cholamidopropyl-dimethylammonio-propanesulfonic acid), 2% NP-40 (nonyl phenoxypolyethoxylethanol-40), 0.002% Bromophenol Blue, 0.5% IPG buffer and 100 mM dithioerythritol. The protein concentration was determined with the 2D Quant kit (GE Healthcare), according to the manufacturer's instructions.

2.4. Two-dimensional gel electrophoresis

Proteins (400 mg) were loaded onto IPG strips (pH 4–7, 11 cm; GE Healthcare) for separation according to their isoelectric point (pI). We started the isoelectric focusing protocol with a 5 h passive rehydration step (0 V), followed by 12 h of active rehydration (50 V), using an isoelectric focusing cell (BioRad, Hercules, CA, USA). The following protocol was used for the remainder of the run (all voltage changes occurred in rapid mode): 500 V for 1 h, 1000 V for 1 h, and 8000 V for 2.5 h. The strips were frozen at −80 °C. Frozen strips were thawed and incubated in equilibration buffer (375 mM Tris-base, 6 M urea, 30% glycerol, 2% SDS [sodium dodecyl sulfate] and 0.002% Bromophenol Blue) for 15 min, first with 65 mM dithiothreitol and then, second with 135 mM iodoacetamide. IPG strips were placed on top of a 12% polyacrylamide gel with a 0.8% agarose solution containing Laemmli SDS electrophoresis (or running) buffer (25 mM Tris-base, 192 mM glycine and 0.1% SDS). Gels were run (Criterion Dodeca; BioRad) at 200 V for 55 min with a recirculating water bath set at 10 °C. Gels were subsequently stained with colloidal Coomassie Blue (G-250) overnight and destained by washing repeatedly with Milli-Q (Millipore, Billerica, MA, USA) water for 48 h. The resulting gel images were scanned with a transparency scanner (model 1280; Epson, Long Beach, CA, USA).

2.5. Gel image analysis

Digitalized images of two-dimensional gels were analyzed using Delta2D (version 4.3; Decodon, Greifswald, Germany) [54]. We used the group warping strategy to connect gel images through match vectors. All images within each metal concentration and time of exposure treatment were fused into a composite image (proteome map), which represents mean volumes for each spot. Spot boundaries were detected within the proteome map and transferred back to all gel images using match vectors. After background subtraction, protein spot volumes were normalized against total spot volume of all proteins in a gel image.

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