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Combined effects of thermal stress and Cd on lysosomal biomarkers and transcription of genes encoding lysosomal enzymes and HSP70 in mussels, *Mytilus galloprovincialis*

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ABSTRACT

In estuaries and coastal areas, intertidal organisms may be subject to thermal stress resulting from global warming, together with pollution. In the present study, the combined effects of thermal stress and exposure to Cd were investigated in the endo-lysosomal system of digestive cells in mussels, *Mytilus galloprovincialis*. Mussels were maintained for 24 h at 18 °C and 26 °C seawater temperature in absence and presence of 50 μg Cd/L seawater. Cadmium accumulation in digestive gland tissue, lysosomal structural changes and membrane stability were determined. Semi-quantitative PCR was applied to reveal the changes elicited by the different experimental conditions in hexosaminidase (hex), β-glucuronidase (gusb), cathepsin L (ctsl) and heat shock protein 70 (hsp70) gene transcription levels. Thermal stress provoked lysosomal enlargement whilst Cd-exposure led to fusion of lysosomes. Both thermal stress and Cd-exposure caused lysosomal membrane destabilisation. hex, gusb and ctsl genes but not hsp70 gene were transcriptionally up-regulated as a result of thermal stress. In contrast, all the studied genes were transcriptionally down-regulated in response to Cd-exposure. Cd bioaccumulation was comparable at 18 °C and 26 °C seawater temperatures but interactions between thermal stress and Cd-exposure were remarkable both in lysosomal biomarkers and in gene transcription. hex, gusb and ctsl genes, reacted to elevated temperature in absence of Cd but not in Cd-exposed mussels. Therefore, thermal stress resulting from global warming might influence the use and interpretation of lysosomal biomarkers in marine pollution monitoring programmes and, vice versa, the presence of pollutants may condition the capacity of mussels to respond against thermal stress in a climate change scenario.

1. Introduction

In marine ectotherms, susceptibility to metal pollution can be strongly modified by seawater temperature through alterations in the rate of biochemical and physiological processes and the stability of biomolecules (Lannig et al., 2006; Sokolova and Lannig, 2008; Sokolova et al., 2012). First, temperature may affect metal chemistry in seawater by changing chemical speciation, pH, solubility, reaction rates, or physical kinetics (Blust et al., 1994; Walther et al., 2002). Temperature rise generally results in added metal bioaccumulation because at high temperatures more bioavailable metal forms are present in the milieu and the metal uptake is enhanced (Wang et al., 2005; Mubiana and Blust, 2006). Second, besides enhancing metal uptake through temperature-dependent physiological and metabolic activation, temperature itself may act as an environmental stressor that may exaggerate the toxic effects of metals through increased mitochondrial damage and oxidative stress, elevated energy demand, impaired ventilatory and circulatory capacities and resulting energy deficiency (Sokolova, 2004; Sokolova and Lannig, 2008). Consequently, global warming may pose a surplus threat for organisms inhabiting moderately polluted areas, and combination of metal exposure and elevated temperatures represents an environmentally realistic scenario in aquatic toxicological research (Lannig et al., 2006). Indeed, interactive effects of toxic metals and elevated temperatures in aquatic ectotherms are being increasingly investigated during the last years (Piano et al., 2004; Sokolova, 2004; Hallare et al., 2005; Cherkasov et al., 2006; Lannig et al., 2006; Ivanina et al., 2009; Micovic et al., 2009; Lee and Jung, 2012; Negri et al., 2013). Particularly, there is a need to investigate the biological responses to short-term extreme elevations in seawater temperature as future climate scenarios envisage that increases in the frequency of extreme events such as heat waves could be more environmentally relevant than long-term trends in average seawater temperatures (IPCC, 2012).

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Biomarkers measured at the molecular or cellular level constitute sensitive early warning tools for measuring biological effects caused by environmental stress, including exposure to pollutants (McCarthy and Shugart, 1990; UNEP/RAMOGE, 1999). Particularly, lysosomal responses in molluscan digestive cells have received much attention as effect biomarkers (UNEP/RAMOGE, 1999; Cajaraville et al., 2000; Marigómez and Baybay-Villacorta, 2003; Izagirre and Marigómez, 2009; Marigómez et al., 2006, 2013a, 2013b; Garmendia et al., 2011a; Brooks et al., 2012). The digestive cells of bivalve molluscs possess a complex endo-lysosomal system that is primarily involved in the uptake and digestion of food materials as well as in xenobiotic accumulation and detoxification (Moore, 1985; Robledo et al., 2006; Izagirre et al., 2008, 2009b). Changes in lysosomal size and reduced membrane stability are the best documented lysosomal responses to environmental pollutants (Regoli, 1992; Marigómez et al., 2005a; Marigómez and Baybay-Villacorta, 2003; Moore et al., 2007; Izagirre and Marigómez, 2009). Likewise, digestive cell lysosomes respond to other sources of environmental stress such as salinity changes, elevated temperature, malnutrition or reproductive stress (Moore, 1976; Tremblay and Pellerin-Massicote, 1997; Izagirre et al., 2008; Garmendia et al., 2010). Concretely, thermal stress induces alterations in lysosomal size and membrane stability in mussels (Moore, 1976; Tremblay et al., 1998; Izagirre and Marigómez, 2009). Moreover, in marine molluscs there is evidence for interactions between thermal stress and biomarkers (Wang et al., 2006; Zhang et al., 2006; Dimitriadis et al., 2012; Negri et al., 2013).

β-Glucuronidase (GUSB) and hexosaminidase (HEX) are the lysosome marker enzymes most commonly used to determine changes in lysosomal size and membrane stability, respectively (Marigómez et al., 2005a; Izagirre et al., 2009a; Izagirre and Marigómez, 2009). HEX and GUSB enzyme activities are characteristic of digestive cells, albeit it might be possible that the enzyme precursors are synthesised in basophilic cells (Dimitriadis et al., 2004; Robledo et al., 2006; Izagirre and Marigómez, 2009). GUSB is present in every compartment of the digestive cell endo-lysosomal system whereas HEX is not so widely distributed and, unlike GUSB, it appears always intimately linked to the lysosomal membrane (Izagirre et al., 2009a). GUSB is a homotetrameric enzyme that plays an important role in the degradation of glucuronid acid containing glycosaminoglycans (Paigen, 1989). HEX is essential lysosomal enzyme that catalyzes the hydrolysis of terminal β-linked N-acetylgalactosamine or N-acetylglucosamine residues from a number of substrates, such as, glycoproteins, glycolipids, and glycosaminoglycans (Gravel et al., 1995).

Cathepsin L (CTSL), a member of the papain superfamily of cysteine proteases, is one of the major lysosomal proteases responsible for cleaving exogenous and endogenous proteins into peptide fragments (Barret and Kirschke, 1981; Katunuma and Kominami, 1987). It plays a major role in intracellular protein catabolism and extracellular matrix turnover, with collagen and elastin among its main substrates (Reynolds, 1969), as well as in protein mobilisation for egg development and maturation in female mussels (Tremblay et al., 1998).

Thermal tolerance in aquatic ectotherms subjected to acute heat stress is critically dependent on the molecular protective mechanisms maintaining cellular integrity such as the synthesis of stress proteins (Pörtner, 2002). Heat shock proteins (HSPs) constitute a family of ubiquitous proteins encoded by genes whose expression is typically activated by thermal stress (Sharf et al., 1998). Among HSPs, the HSP70 family appears to be most conserved throughout evolution and is widely distributed in the animal kingdom (Sanders, 1993). In molluscs, protein expression studies revealed that HSP70s appear after heat shock (Buckley et al., 2001; Piano et al., 2002). On the other hand, although HSPs were originally identified via their up-regulation in response to heat stress, they also respond to a great variety of stressors (e.g., temperature, hypoxia, pH, salinity, pollution) and perform chaperoning roles assisting polypeptide folding during protein synthesis (Hochachka and Somero, 2002; Ioannou et al., 2009; Anestis et al., 2010).

The aim of the present study was to elucidate whether thermal stress can influence the use and interpretation of lysosomal biomarkers in mussels exposed to Cd and vice versa whether Cd exposure may condition the capacity of mussels to respond against thermal stress. For this purpose, the combined effects of thermal stress and Cd-exposure on lysosomal biomarkers and on the transcription of genes encoding lysosomal enzymes (HEX, GUSB and CTSL) and the *hsp70* gene were investigated in the digestive gland of mussels, *Mytilus galloprovincialis*, under controlled laboratory conditions. As a whole, this study provides insights into the mechanisms of interactions of temperature and metal stress on the lysosomal system of marine bivalves and thus are important for understanding of the physiology of intertidal species during the global change in polluted estuaries and coastal zones.

2. Materials and methods

2.1. Experimental procedure

Mussels ($n = 180$; 3.5–4.5 cm shell length), *M. galloprovincialis*, were collected at spring tide from the lowest tidemark level (0–1 m) in Plentzia (Biscay Bay, N 43°25′17″; W 2°56′51″) in July 2007. After routine histological examination of the gonad they were determined to be at post-spawning gametogenic stage. The month range of variation in surface seawater temperature at regional scale (Basque Coast) was 16–20 °C (Goikoetxea et al., 2009). Mussels were transferred to the laboratory immersed in aerated clean seawater and kept unfed at 18 °C for 18 h under laboratory conditions. Then, four experimental conditions were established for 24 h under constant aeration: seawater at 18 °C (room temperature) and 26 °C (using a thermostat heater), with Cd (50 μg Cd/L; add as chloride) and without Cd. For each experimental treatment 3 replicates were used with 15 mussels each in 2.5 L recipients. Mussels were fed a commercial food mixture (Korall fluid, JBL Gmbtt & Co., Germany) at the beginning of the 24 h experiment. After 24 h treatment, digestive gland was dissected out and processed in different ways. Since replicates were used only for experimental purposes and the conditions in the 3 replicates of each treatment were identical, samples were mixed ($N = 45$ per treatment group). Thus, 10 digestive glands per treatment were frozen in liquid nitrogen in cryovials with RNA-later (Invitrogen Life Tech., USA) solution for gene transcription studies. Two pools of 5 digestive glands were rapidly placed on a plastic chuck aligned in a straight row across the centre, put into cryovials and directly frozen by immersion in liquid nitrogen. Once frozen, cryovials were stored at −80 °C until required for analyses. Additional 15 (5×3 pools) digestive glands per treatment were dried at 137 °C for 48 h to be further processed for chemical analysis of Cd.

2.2. Chemical analysis (atomic absorption spectrophotometry)

Dried digestive gland pools (3 per treatment) were crushed in a mortar to facilitate digestion. Then, dried tissues were digested in concentrated nitric acid, diluted to 0.1 M nitric acid, and analysed by atomic absorption spectrophotometry (Fl-AAS; Perkin Elmer AAnalyst 100), with simultaneous background corrections and a sensitivity of 0.249 mg Cd/L. Merck standard solutions and certified reference materials were diluted in 0.1 M nitric acid for AAS calibration. Cd concentration was expressed as μg Cd/g digestive gland dry-wt.