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Effects of elevated temperature and cadmium exposure on stress protein response in eastern oysters *Crassostrea virginica* (Gmelin)

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ABSTRACT

Stress proteins such as heat shock proteins (HSPs) and metallothioneins (MTs) play a key role in cellular protection against environmental stress. Marine ectotherms such as eastern oysters *Crassostrea virginica* are commonly exposed to multiple stressors including temperature and pollution by metals such as cadmium (Cd) in estuaries and coastal zones; however, the combined effects of these stressors on their cellular protection mechanisms are poorly understood. We acclimated *C. virginica* from populations adapted to different thermal regimes (Washington, North Carolina and Texas) at a common temperature of 12 °C, and analyzed their expression of MTs and HSPs (cytosolic HSP69, HSC72–77, HSP90 and mitochondrial HSP60) in response to the combined acute temperature stress and long-term Cd exposure. Overall, HSP and MT induction patterns were similar in oysters from the three studied geographically distant populations. HSP69 and MTs were significantly up-regulated by Cd and temperature stress implying their important role in cellular stress protection. In contrast, HSC72–77, HSP60 and HSP90 were not consistently induced by either acute heat or Cd exposure. The induction temperature for MTs was higher than for HSP69 (>28 °C vs. 20 °C, respectively), and MTs were more strongly induced by Cd than by temperature stress (to up to 38–94-fold compared by 3.5–7.5-fold, respectively) consistent with their predominant role in metal detoxification. Notably, heat stress did not result in an additional increase in metallothionein expression in Cd-exposed oysters suggesting a capacity limitation during the combined exposure to Cd and temperature stress. Levels of HSP69 and in some cases, HSC72–77 and HSP90 were lower in Cd-exposed oysters as compared to their control counterparts during heat stress indicating that simultaneous exposure to these two stressors may have partially suppressed the cytoprotective upregulation of molecular chaperones. These limitations of stress protein response may contribute to the reduced thermotolerance of oysters from metal-polluted environments.

1. Introduction

Pollution of aquatic environments by trace metals including cadmium is a world-wide problem due to the persistency and continuing accumulation of metals in the environment (GESAMP, 1987; de Mora et al., 2004; Hyun et al., 2006). Cadmium (Cd) is one of the most toxic metals found in coastal zones and estuaries where it is predominantly released by human activities such as smelting, mining, battery manufacturing, and pigment and plastic production (GESAMP, 1987; Pinot et al., 2000). In ectotherms (that constitute >99% of species in aquatic realms), susceptibility to metal pollutants can be strongly modified by the environmental temperature due to its direct effects on all biochemical and physiological reactions (reviews in: Hochachka and Somero, 2002; Gordon, 2005; Sokolova and Lannig, 2008). Earlier studies have shown that moderately elevated temperatures exaggerate toxic effects of Cd on aquatic ectotherms through increased mitochondrial damage and oxidative stress, elevated energy demand, impaired ventilatory and/or circulatory capacities and resulting energy deficiency (review in: Sokolova and Lannig, 2008). However, it is not known whether Cd exposure can also affect the response to and/or tolerance of temperature extremes in marine ectotherms. According to the hierarchical model of the mechanisms of thermal tolerance in aquatic ectotherms (review in: Pörtner, 2002), survival under the conditions of acute heat stress is critically dependent on the molecular protective mechanisms maintaining cellular integrity such as the expression of stress proteins, and alterations of the capacity of these cytoprotective systems can have serious implications for the whole-organism survival under the extreme temperature conditions.

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Two important groups of stress proteins – heat shock proteins (HSPs) and metallothioneins (MTs) – play a key role in cellular protection against toxic metals and temperature stress (Bauman et al., 1993; Andrews, 2000; Amiard et al., 2006) and are the focus of the current study. Metallothioneins are low molecular weight cysteine-rich proteins with prominent metal-binding and redox capacities (Amiard et al., 2006; Bauman et al., 1993; Coyle et al., 2002; Palmiter, 1998). Their major function involves regulation of intracellular levels of essential and non-essential metals (including Cd) and metal detoxification (Roesijadi, 1996; Palmiter, 1998; Klaassen et al., 1999; Amiard et al., 2006), while secondary roles include antioxidant defense, protection against xenobiotics, inflammation and infection through free radical scavenging (Tamai et al., 1994; DeMoor et al., 2001; Van Cleef-Tödt et al., 2001; Coyle et al., 2002; Piano et al., 2004). HSPs also play an important role in protection against multiple stressors (including heat stress, toxic metals, ionizing and UV radiation and others) and act as molecular chaperones that assist in ATP-dependent folding and stabilization of stress-damaged proteins (Parsell and Lindquist, 1993; Somero, 1995; Hofmann et al., 2002; Boutet et al., 2003; Piano et al., 2004; Hofmann, 2005; Moraga et al., 2005). Cytosolic chaperones HSP70 and HSP90 are among the most abundant cellular proteins protecting from stress-induced damage. HSP70 is the family of universal cytosolic chaperones involved in folding of reparably damaged proteins and in degradation of those that are damaged beyond repair (Mayer and Bukau, 2005). HSP90 is another general cytosolic chaperone orchestrating the folding of many proteins; however, HSP90 alone is insufficient to assist refolding of partially denatured proteins, and requires other chaperones such as HSP70 to complete this task (Csermely et al., 1998; Mayer and Bukau, 2005). In contrast, HSP60 is predominantly found in mitochondria and chloroplasts assisting with the protein folding and stress protection in these organelles (Cechetto et al., 2000). Both metallothioneins and HSPs are essential for survival of an organism exposed to toxic metals and heat stress (Parsell and Lindquist, 1993; Somero, 1995; Roesijadi, 1996; Klaassen et al., 1999). However, it is not known how the concomitant exposure to these combined stressors affects expression of MTs and HSPs in aquatic ectotherms.

The goal of this study was to analyze the induction patterns of MTs and HSPs (including cytosolic HSP69, HSC72–77, HSP90 and mitochondrial HSP60) in response to combined temperature and Cd stress in a model marine ectotherm, eastern oyster *C. virginica*. We tested whether Cd exposure alters stress protein expression during acute heating in oysters and compared expression patterns of MTs and HSPs in oysters from populations adapted to different climates in order to assess potential geographical variation in the studied parameters. Oysters are commonly exposed to metals including Cd and to temperature stress in their habitats and thus can serve as a useful model to address these questions. They have an ability to accumulate Cd burdens exceeding the environmental levels by orders of magnitude, making them susceptible to the toxic effects of Cd as well as important vectors of Cd transfer to the higher levels of the food chain (Roesijadi, 1996; Frew et al., 1997; Pigeot et al., 2006). Like all intertidal organisms, oysters also can experience rapid and extreme temperature fluctuations, with a change in body temperature by up to 10–20 °C within a few hours during the diurnal/tidal cycles and even more dramatic changes (from 0 to 35–40 °C) over the longer (seasonal) time span (Sokolova et al., 2000; Helmuth et al., 2002; Cherkasov et al., 2007). Investigations of the interactive effects of temperature and metal stress on cytoprotective mechanisms of oysters can provide a better understanding of physiological and cellular mechanisms of stress response in aquatic ectotherms, and the factors setting limits to their tolerance in the face of multiple stressors in polluted estuaries.

2. Materials and methods

2.1. Animal collection and maintenance

Oysters (*C. virginica*) were obtained from J & B Aquafood (Jacksonville, NC, USA), Taylor Shellfish Farms (Totten Inlet, Shelton, WA, USA), and Jeri's Seafood Inc. (Smith Point, TX, USA) in winter–early spring 2007. Oysters were shipped within 24–48 h to the University of North Carolina at Charlotte and placed in recirculated aerated tanks with artificial seawater (ASW) (Instant Ocean®, Kent Marine, Acworth, USA) at 12 ± 1 °C and 30‰ which was close to the temperature and salinity of their habitats at the time of collection. It is worth noting that although our collections were performed in winter when surface water temperatures were similar in the three studied sites, their seasonal thermal regimes are different. Mean monthly surface water temperature varies between 12–15 °C in winter and 28–30 °C in summer near Smith Point, TX, and between 6–12 °C in winter and 28–32 °C in summer in Stump Sound, NC, respectively (IDARS NOAA at http://www.nodc.noaa.gov/dsdt/index.html; Cherkasov et al., 2007). The surface water temperature averages 8–10 °C during the winter months and 12–13 °C in summer near the study site in Totten Inlet, WA (IDARS NOAA at http://www.nodc.noaa.gov/dsdt/index.html). All three collection sites are used for commercial oyster culture for human consumption and have low background levels of metals and other pollutants.

Oysters were allowed to acclimate at 12 ± 1 °C and 30‰ for 2–3 weeks and fed *ad libitum* on alternate days with a commercial algal blend (2 mL per oyster) containing *Nannochloropsis*, *Tetraselmis*, and *Isochrysis* spp. with a cell size of 2–15 μm (PhytoPlex; Kent Marine, Acworth, GA, USA) or *Nannochloropsis oculata*, *Phaeodactylum tricornutum* and *Chlorella* with a cell size of 2–20 μm (DT's Live Marine Phytoplankton, Premium Reef Blend, Sycamore, IL, USA). After the preliminary acclimation, half of the tanks were randomly selected, and Cd (as $CdCl_2$) was added to the nominal concentration of 50 $\mu g\,L^{-1}$. The remaining tanks were used as controls. Oysters were exposed to Cd (50 $\mu g\,L^{-1}$) or clean ASW (controls) for 45–50 days at 12 °C prior to subsequent experiments. To avoid pseudoreplication, two tanks were set for each combination of Cd exposure (i.e. control and 50 $\mu g\,L^{-1}$) and study population (WA, NC or TX), and oysters were haphazardly sampled from these tanks for each experiment. It is worth noting that Cd concentrations in our experimental exposures were at the upper end of Cd concentrations found in polluted estuaries (Crompton, 1997). However, our previous studies have shown that up to 60 days of exposure under these conditions result in physiologically relevant tissue burdens of Cd similar to those found in oysters from polluted estuaries (Sokolova et al., 2005; Cherkasov et al., 2006 and references therein).

2.2. Experimental exposures

Because transcriptional and translational responses to environmental stress can be delayed due to the time needed to synthesize mRNAs from target genes and to translate them into proteins, we performed a pilot experiment to determine the time course of mRNA and protein expression in response to acute heating and to identify the time window of the maximum stress response for HSPs and MTs (see below Section 2.3 for the measurement techniques). Oysters were exposed to 40 °C for 1 h and allowed to recover for up to 21 days at their acclimation temperature. Exposure to 40 °C is an acute stressor which is strong enough to elicit significant stress response while remaining in the environmentally relevant range for intertidal organisms such as oysters (Hamdoun et al., 2003; Helmuth et al., 2002; Cherkasov et al., 2007). Analysis of expres-