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CHARACTERIZING THE EFFECTS OF HEAVY METAL AND *VIBRIO* EXPOSURE ON HSP70 EXPRESSION IN *CRASSOSTREA GIGAS* GILL TISSUE

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ABSTRACT The Pacific oyster, *Crassostrea gigas,* is an intertidal bivalve mollusc that inhabits several continents. Similar to other shellfish, the Pacific oyster is considered an important bioindicator species. One way to assess environmental perturbations is to examine an organism's stress response. Molecular chaperones, including heat shock proteins, are common targets when evaluating an organism's response to environmental stress. In this study, oysters were exposed to copper and the bacterium *Vibrio tubiashii* to characterize how these environmental stressors influence Hsp70 gene and protein expression. Bacterial exposure did not affect Hsp70 expression, whereas copper exposure changed both transcript and protein levels significantly. Interestingly, copper exposure increased gene expression and decreased protein levels when compared with controls. The dynamics of Hsp70 regulation observed here provide important insight into heavy metal exposure and heat shock protein levels in oysters, highlighting considerations that should be made when using Hsp70 as an indicator of an organism's general stress response.

KEY WORDS: *Crassostrea gigas,* Pacific oyster, Hsp70, gene expression, protein expression, copper, *Vibrio*

INTRODUCTION

Perturbations in environmental conditions can alter physiological processes, and measuring these changes can provide information with respect to an organism's response to stress. A common means of characterizing a physiological response to environmental change is by measuring changes in gene expression levels, because messenger RNA (mRNA) expression is considered directly related to corresponding protein levels (Bierkens 2000). However, protein expression does not always correlate with mRNA levels (Gygi et al. 1999, Piña et al. 2007). Consequently, analysis of stress response pathways that do not account for both transcriptional and translational mechanisms may not represent the organism's response accurately (Greenbaum et al. 2003).

Heat shock proteins (Hsp) are a common focus in assays used to establish conditions of physiological and environmental stress (Downs et al. 2001, Piña et al. 2007, Franzellitti et al. 2010, Roberts et al. 2010). Heat shock proteins are involved in a variety of stress-related processes, including thermal stress, immune response, and apoptosis (Feder & Hofmann 1999, Roberts et al. 2010). The most highly conserved family of heat shock proteins is the Hsp70 family (Sanders et al. 1994). Hsp70s are molecular chaperones involved in the folding and unfolding of newly translated proteins, and proteins that have been damaged from various forms of cellular stress (Feder & Hofmann 1999). Hsp70 was originally characterized in *Crassostrea gigas* by Gourdon et al. (2000), followed by further sequence identification and characterization by Boutet et al. (2003). Hsp70 is one of the most targeted proteins for studying the stress response in shellfish (Fabbri et al. 2008).

Oysters, like other bivalves, are sessile organisms that are continually filtering water, and they often bioaccumulate toxins, making them ideal bioindicator species (Rittschof & McClellan-Green 2005, Fabbri et al. 2008, Morley 2010). Although studies have documented Hsp70 accumulation in response to individual stressors such as temperature and heavy metals (Clegg et al. 1998, Boutet et al. 2003), few studies have characterized Hsp70 in organisms that are subjected to multiple stressors. Combinations of environmental contaminants can act synergistically, the effects of which can be greater than the sum of the individual stressors combined (Anderson et al. 1998). Because combinations of stressors are more representative of natural ecosystems, characterizing physiological stress response in the presence of multiple stressors is relevant to understanding stress response physiology (Anderson et al. 1998, Gupta et al. 2010).

The primary objective of the current study was to characterize Hsp70 activity at the transcript and protein levels in *C. gigas* when exposed to copper and bacteria. Copper is one of several heavy metal contaminants found in aquatic environments (O'Connor & Lauenstein, 2005). The bacterial pathogen *Vibrio tubiashii* is a reemerging bacterial pathogen of shellfish on the west coast of North America (Elston et al. 2008). This study provides insight into heavy metal exposure in shellfish and heat shock protein activity, and highlights considerations that should be made when using Hsp70 as an indicator for an organism's general stress response.

MATERIALS AND METHODS

Experimental Design

Pacific oysters were collected from the University of Washington's field station at Big Beef Creek, WA, in November 2010. A total of 32 oysters were collected with a mean length of 112.3 mm. After 4 days of acclimation in 12°C seawater, oysters were assigned randomly to 4 treatment groups ($n = 8$). One group received a dose of copper(II) sulfate (33 mg/L copper ion concentration) for 72 h. A second group of oysters were subjected to a *V. tubiashii* (Strain ATTC 19106) bath exposure at 7.5×10^5 CFU/mL for 24 h. A third group of oysters was subjected to a combined copper and *V. tubiashii* exposure. Oysters in this group were first exposed to copper for 48 h followed by 24 h of copper and *V. tubiashii* combined. The fourth group of oysters served as a control and was held in seawater for the duration of the experiment. The bacterium *V. tubiashii* was cultured overnight at 37°C in 1 L LB broth, plus an additional 1% NaCl. Cells were harvested by centrifugation at 4,200 rpm for 20 min. Pelleted bacteria were then resuspended

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in 0.22-μm filter sterilized seawater. Immediately after treatments, gill tissue was removed and stored at –80°C for subsequent RNA and protein extraction.

cDNA Synthesis and Quantitative PCR Analysis

RNA from all gill tissue samples (~25 mg) was extracted using TRI Reagent (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer's protocol. RNA samples were treated with the Turbo DNA-free Kit (Ambion Inc., Foster City, CA) per the manufacturer's recommended standard protocol to remove potential genomic DNA carryover. RNA (1 μg) was reverse transcribed using M-MLV reverse transcriptase (Promega, Madison, WI) according to the manufacturer's protocol. For gene expression analysis, primers were designed for Hsp70 (GenBank accession no. AJ318882) (hsp70fw:TGGCAACCAATCGCAAGGTGAG; hsp70rv: CCTGAGAGCTTGAGGACAAGGT) using Primer3 software (Rozen & Skaletsky 2000). Quantitative PCR (qPCR) reactions were carried out in a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA). Each 25-μL reaction contained 1× Immomix Master Mix (Bioline USA Inc., Boston, MA), 0.2 μM of each primer, 0.2 μM Syto-13 (Invitrogen, Carlsbad, CA), 2 μL cDNA, and sterile water. Thermocycling conditions included an initial denaturation (10 min at 95°C), followed by 40 cycles of 15 sec at 95°C, 15 sec at 55°C, and 30 sec at 72°C, with fluorescence measured at the end of the annealing and extension steps. After qPCR, melting curve analysis was performed by increasing the temperature from 65°C to 95°C at a rate of 0.2°C/sec, measuring fluorescence every 0.5°C. All samples were run in duplicate. Analysis of qPCR data was carried out based on the kinetics of qPCR reactions (Zhao & Fernald 2005) and normalized to elongation factor 1 alpha expression (ef1fw: AAGGAAGCTGCTGAGATGGG; ef1rv: CAGCACAGTCAGCCTGTGAAGT) (GenBank accession no. AB122066). DNased RNA was used as a template to ensure that no genomic DNA carryover was present. Data are expressed as fold increases over the minimum.

Protein Isolation and Western Blot Analysis

Protein was extracted from gill tissue using CellLytic Cell Lysis Reagent tissue (Sigma, St. Louis, MO) containing protease inhibitor cocktail (Sigma) at a ratio of 1:20 (1 g tissue/20 mL reagent). The levels of Hsp70 protein were assessed by Western blot analysis using antiheat shock protein 70 monoclonal antibody (3A3; product no. MA3-006, Pierce, Thermo Fisher Scientific, Rockford, IL). Total protein was subjected to gel electrophoresis using 4–20% Precise Protein Gels (Precise, Thermo Fisher Scientific). Gels were transferred to nitrocellulose membranes, blocked, and incubated with diluted (1:3,000) primary Hsp70 antibody. Membrane incubations and visualization were carried using the WesternBreeze Chromogenic Kit-AntiMouse (Invitrogen, Carlsbad, CA). Integrated density values were calculated using Image J (Abramaoff et al. 2004). Values of background densities on SDS-PAGE gels were calculated and used to normalize values based on protein band density.

Statistical Analysis

Two-way ANOVAs were carried out to determine significant differences in Hsp70 mRNA expression and protein levels ($P < 0.05$; SPSS v18). Because no effect of *V. tubiashii* was detected for either Hsp70 mRNA expression or protein levels, data from *V. tubiashii*-exposed oysters were combined with control oysters and data from *V. tubiashii*- and copper-exposed oysters was combined with the copper-exposed oysters, and a *t*-test was conducted to determine the effect of copper exposure on Hsp70 expression ($P < 0.05$).

RESULTS

Exposure

Hsp70 gene and protein levels were not significantly different in gill tissue from oysters exposed only to *V. tubiashii* when compared with controls (data not shown). Furthermore, mRNA expression and protein levels in gill tissue from oysters exposed to *V. tubiashii* in combination with copper were not significantly different from oysters exposed to copper alone. Because there was no influence of *V. tubiashii* exposure on Hsp70 mRNA expression or protein levels, subsequent analyses and data presented from copper-exposed oysters refer to those oysters exposed to copper only ($n = 8$), and the group of oysters exposed to copper and *V. tubiashii* ($n = 8$).

Copper Exposure

Copper exposure altered both gene and protein expression significantly. Specifically, expression of Hsp70 mRNA was significantly higher in copper-treated oysters compared with control individuals (Fig. 1A). Protein expression was decreased significantly in gill tissue from oysters exposed to copper compared with controls (Fig. 1B).

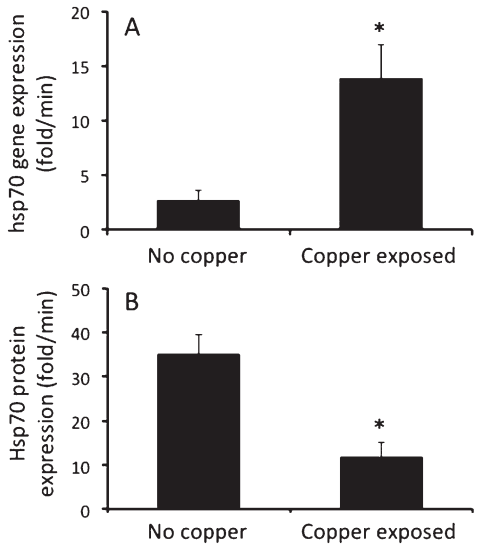

Figure 1. (A, B) Quantification of hsp70 mRNA (A) and protein (B) in oysters held in seawater or in 33 mg/L copper. Expression values are presented as fold change over the minimum expression value ± SE. "No copper" expression values include both control and *V. tubiashii*-exposed animals whereas "copper exposed" includes both copper only and copper and *V. tubiashii*-treated animals because *V. tubiashii* exposure was shown to have no significant effect on hsp70 expression by ANOVA. Asterisks denote a significant difference in hsp70 expression compared with the no-copper treatment ($P < 0.05$).

DISCUSSION

Although Hsp70 expression has received considerable attention as an indicator of stress in shellfish, there are limited studies that characterize both gene and protein Hsp70 expression. This study demonstrates that exposure to copper influences Hsp70 gene and protein expression in oysters. Furthermore, exposure to copper resulted in discordant expression of Hsp70 mRNA and protein.

Oysters used in this study were exposed to copper (33 mg/L), which resulted in a significant increase in Hsp70 mRNA expression in gill tissue. Gill tissue was selected for analysis because it is rich in hemocytes and is exposed to water and waterborne contaminants. In addition, gill tissue has been identified previously to accumulate the highest levels of copper compared with other tissues in oysters (Han & Hung 1990). The level of copper used here was more than that measured in typical commercial areas or marinas (e.g., Schiff et al. 2007), although it was similar to levels measured near mining operations (e.g., Robertson & Kirsten 2003). Our results are consistent with studies that have used lower levels of copper. In *Argopecten purpuratus,* Zapata et al. (2009) observed that exposure of copper as low as 2.5 μg/L for 8 days showed significant increases in Hsp70 mRNA. The zebra mussel *Dreissena polymorpha* exposed to copper had significantly increased expression of Hsp70 mRNA in gill tissue after 24 h of exposure that returned to control levels after 7 days (Navarro et al. 2011). A study of *Fenneropenaeus chinensis* showed a significant increase in Hsp70 mRNA in shrimp exposed to 20 μg/L copper after 24 h of exposure; however, 72 h of exposure actually decreased expression levels (Luan et al. 2010). The decrease in gene expression level in *F. chinensis* could be attributed to the toxicity threshold being exceeded, impairing general biological processes including transcription. In the current study, increased expression of Hsp70 mRNA after 72 h of exposure indicates resilience in the oyster's ability to respond to high levels of copper exposure. Assuming that Hsp70 gene expression would no longer be affected by a specific environmental stressor, an interesting direction for future research would be to examine this hypothesis across numerous stressors. In addition, it is likely that resilience and the physiological response will vary with developmental stage.

Interestingly, expression levels of Hsp70 protein were significantly less in gill tissue of copper-exposed oysters compared with controls. These results are consistent with other studies of *C. gigas* in which decreased levels of Hsp70 protein were observed after 24 h, reaching their lowest level after 3 days in oysters exposed to lower levels of copper (Boutet et al. 2003). However, in other bivalves, copper exposure has resulted in an increase in Hsp70 protein concentration. Zebra mussels exposed to 100–500 μg/L copper had increased Hsp70 protein levels whereas no change was detected in lower doses (Clayton et al. 2000). Protein levels for Hsp70 were also elevated in *Mytilus edulis* after 7 days of exposure to copper (Sanders et al. 1994). In *Chamelea gallina,* a concentration-dependent regulation of protein expression was observed in which protein concentrations increased in the presence of low copper concentrations and decreased in high copper treatments (>5 mg/mL) (Rodriquez-Ortega et al. 2003). Changes in Hsp70 protein levels in shellfish exposed to copper appears to be taxon and dose dependent. In oysters, relatively low levels of copper (Boutet et al. 2003) and higher doses (the current study) both resulted in decreased Hsp70 protein, which could indicate that across a range of copper exposures, Hsp70 proteins in oysters are functioning properly to modify the structure of damaged proteins. Within the cytoplasm, Hsp70 often forms a multichaperone complex with Hsp90, Hsp40, and the monomeric transcription factor HSF-1. When unfolded or misfolded proteins are present, this complex disassociates so that the heat shock proteins can prevent the denaturation of proteins or remove degraded proteins (Bierkens 2000). The now-dissociated HSF-1 eventually facilitates increased Hsp70 transcription. Thus, at least in oysters, the decreased protein concentration observed could be associated directly with the dissociation of the multichaperone complex and shortened residence time of Hsp70 proteins as they actively prevent denaturation or remove proteins. Likewise, the translation of new Hsp70 protein is not translated at a rate to compensate for the decrease. The difference across species could therefore be associated with varying rates of transcription and translation. Other important considerations are that copper exposure could damage Hsp70 proteins themselves or overall translational machinery (Lewis et al. 2001), which would result in lower protein levels.

This study illustrates that environmental stressors can influence Hsp70 gene and protein expression in a discordant fashion. We did not observe an effect of environmentally relevant levels of *V. tubiashii* (Elston et al. 2008) on Hsp70 expression, and presumably the bacterial exposure did not impact the physiological response to copper significantly. The influence of copper on Hsp70 regulation in oysters appears to be different compared with closely related species, which could offer an interesting system to study Hsp70 dynamics in greater detail. Understanding the mechanisms responsible for this apparent difference in the sensitivity of Hsp70 regulation between species could help us understand mechanisms underlying species resilience more completely. These findings highlight important considerations that should be taken when using Hsp70 as an indicator of environmental stress and the associated physiological response.

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