Stressed-Out Lobsters: Crustacean Hyperglycemic Hormone and Stress Proteins

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SYNOPSIS. Organisms in natural habitats must frequently respond to changes in their environments through various physiological mechanisms. My laboratory has developed several methods for the quantification of stress in crustaceans. An ELISA was developed for the crustacean hyperglycemic hormone (CHH) from the American lobster (Homarus americanus). It is sensitive to as little as 0.2 fmol of peptide. Increases in hemolymph CHH were observed under conditions of acute hypoxia, elevated temperature, and altered salinity. In addition, elevated CHH concentrations were observed in Norway lobsters (Nephrops norvegicus) that were parasitized with the dinoflagellate Hematodinium sp.

Stress proteins, also known as heat-shock proteins (HSPs), comprise a highly conserved class of proteins that display elevated transcription during periods of stress. Using homologous molecular probes, my collaborators and I have examined the influence of heat-shock, osmotic stress, and the molt cycle upon HSP expression at the protein and mRNA levels. We observed a significant elevation in HSP mRNA expression after 1 hr of heat-shock or after 0.5 hr of osmotic stress. When comparing claw and abdominal muscles during molting, we observed a tissue-specific HSP response. Quantification of these different stress responses may serve as early indicators of the degradation of environmental health.

INTRODUCTION

Animals display a variety of stress responses when their regulated physiological systems are extended beyond their normal range. Partial or complete failure of the homeostatic response may lead to increasing physiological disturbance and ultimately death. Biochemical indicators of such stress responses may be more sensitive than physiological responses and hence are more useful in the assessment of the overall health of an animal. In this review of data obtained from my laboratory and those of my collaborators, I focus on alterations in the concentration of circulating crustacean hyperglycemic hormone (CHH) and the induction of stress proteins.

Hyperglycemia as a response to various kinds of stress has been observed in decapod crustaceans (Telford, 1968). Regulation of hemolymph glucose is mediated by the release of CHH that is synthesized in the eyestalk X-organ and stored prior to release from the sinus gland (for review see Böcking et al., 2002). We (my collaborators and I) developed an enzyme-linked immunosorbent assay (ELISA) for CHH as a tool for the quantification of various acute environmental stresses in American lobsters (Homarus americanus). These stresses included hypoxia, thermal stress, and salinity stress (Chang et al., 1998). We also examined the effects of the stresses imposed by parasitism upon CHH concentrations in the Norway lobster (Nephrops norvegicus; Stentiford et al., 2001).

Another response to environmental and physiological stress is the production of stress or heat shock proteins (HSPs). The primary function of HSPs is to act as molecular chaperones, promoting the initial folding of other proteins at the ribosome and the refolding of unfolded proteins when they are partially denatured (Nelson et al., 1992), though other functions have been ascribed to HSPs (Pratt, 1997). Induction or elevated expression of HSPs has been shown to occur in response to a number of stresses in many organisms (see review by Feder and Hofmann, 1999). In the latter part of this limited review, I discuss our gene expression studies with lobster HSPs.

These studies may provide general indicators of environmental health. In addition, the CHH ELISA may be useful for the quantification of circulating CHH levels following exposure to endocrine disrupting compounds. Thus our studies encompass two of the themes of this symposium.

MATERIALS AND METHODS

Hemolymph CHH

Development of the ELISA has previously been described (Chang et al., 1998, 1999). Briefly, the primary antibody was made in a rabbit against HPLC-purified lobster CHH-A (Tensen et al., 1991). It was used to coat multiecell modules. Hemolymph samples or standards were allowed to incubate overnight. The biotinylated secondary antibody was added and then followed with streptavidin–peroxidase and 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid (color reagent). The optical densities of the solutions in the wells were quantified at 405 nm. Crayfish (Orconectes limosus) CHH was used as a standard. Previous calibrations comparing the cross-reactivity of HPLC-purified lobster and crayfish CHH allowed an accurate determination of lobster CHH in the ELISA.

Prior studies demonstrated that N. norvegicus CHH could also be detected using this antibody and assay (Stentiford et al., 2001). Only relative changes in circulating CHH could be determined due to the lack of purified standard N. norvegicus CHH.
pleton (1995) characterized highly infected lobsters (Stages III and IV) as having >76% of hemolymph cells consisting of parasites.

Tissue HSPs–western blot

Abdominal muscle, embryos, and larval tissues from *H. americanus* were homogenized in a hypotonic cell lysis buffer (10 mM HEPES, 10 mM Tris HCl, 1 mM EDTA, 0.25 M sucrose, pH 7.2) containing a protease inhibitor cocktail (Sigma P2714; 10 ml of buffer is added to the stock vial to make a 10× stock. The stock is further diluted to 1× when mixed with buffer). Following centrifugation (10 min, 15,000 g), aliquots of the resulting supernatants were taken for protein assay (Bio-Rad DC protein assay) and also combined (equal volumes) with 2× SDS sample buffer for SDS-PAGE (polyacrylamide gel electrophoresis) (Laemmli, 1970). SDS-PAGE samples were heated for 5 min at 100°C, and loaded onto 18-well, 12.5% pre-cast polyacrylamide gels (Bio-Rad Criterion). Loading was based on equal protein concentrations (30 μg/lane). HSP positive control (standard) proteins were loaded onto each of the gels (50 ng of recombinant human HSP70 [Stressgen SSP-770]; 20 μg of total protein from heat-shocked HeLa cell lysates [Stressgen LYC-HL101]). Proteins were electrophoresed with 200 V (Bio-Rad Criterion Cell) until the dye front ran off (resed with 200 V (Bio-Rad Criterion Cell) until the base of the last walking leg. Controls were matched siblings that were left immersed at 13°C and sampled at the same time points. Lobsters ranged in wet weight from 100 to 145 g. Means ± SD are shown. Control data are represented by the triangles and dashed line (n = 8). Asterisks indicate significant differences from immersed controls at P < 0.01 (**) and at P < 0.001 (***) Modified from Chang et al. (1998).

**RESULTS**

**Hemolymph CHH**

We observed that emersion is a potent stimulator for the elevation of hemolymph CHH (Chang et al., 1998). Emersion results in hypoxia in many aquatic crustaceans. Figure 1 shows that CHH concentrations increase from resting values of 4.0 fmol/ml to 168.1 fmol/ml after 4 hr of emersion. Although handling stress slightly increases CHH, the additional stress of emersion is significantly above the handling stress observed in immersed controls (from 11.3 to 20.6 fmol/ml) held at the same temperature (13°C) as the emersed lobsters.

Thermal stress caused an increase in hemolymph CHH. Figure 2 shows that a 10°C elevation in temperature to 23°C caused a significant increase in CHH relative to ambient (13°C) controls. The response lasted for only about 2 hr. The CHH levels of the heated lobsters were close to the controls after 4 hr of constant heat. No significant changes in hemolymph CHH were observed following a 5°C temperature elevation nor were changes seen during cold stress (data not shown).

Both hyposalinity (50%) and hypersalinity (150% seawater) resulted in significant alterations in hemolymph CHH after 2 hr. This elevation in CHH relative to the controls was not significant at later time points (Fig. 3). As seen above, there was a slight elevation in CHH due to handling stress.

The concentration of CHH in the plasma of uninfected *N. norvegicus* was 32.2 ± 7.8 fmol/ml (mean ± SD). This is contrasted by the levels in lobsters that were highly infected with the dinoflagellate *Hematodinium* sp. The concentration of CHH in the plasma...
HSP90 mRNA was observed (Fig. 5A, C; after 2 hr of heat-shock, a significant elevation in both hypo- and hyper-osmotic stress (Fig. 6A). HSP70 cle HSP70 mRNA levels were significantly induced by heat-shock responses could be quantified. Following 1 hr of heat-shock, we observed a significant induction of abdominal muscle HSP70 mRNA levels over control levels at 1 hr ($P < 0.01$) and in 50% seawater ($P < 0.01$) time points of the hypo-osmotic treatment. In 50% seawater, HSP90 mRNA levels were significantly greater than control levels by 0.5 hr of exposure ($P < 0.001$). HSP90 mRNA levels also remained significantly elevated at the 1 hr ($P < 0.001$) and 2 hr ($P < 0.01$) time points of the hypo-osmotic treatment. In 150% seawater, abdominal muscle HSP90 mRNA levels were significantly increased over control levels by 0.5 hr of exposure ($P < 0.001$) and remained elevated at the 1 hr ($P < 0.01$) and 2 hr ($P < 0.01$) time points.

Preparations for and recovery after molting are physiological challenges for crustaceans. As stated by Herrick (1911) “the molting act is a continually recurring crisis in the life of the decapod crustacean for it is both dangerous and expensive.” To determine if molting induced HSPs, we injected physiological amounts of the molting hormone, 20-hydroxyecdysone, into juvenile lobsters. We observed 2-fold increases in HSP90 mRNA in the hepatopancreas 48 hr later (Chang et al., 1999).

In another experiment, lobsters were sacrificed at different stages of the molt cycle and their abdominal and claw muscles were extracted for northern analyses. HSP90 gene expression was significantly induced in levels by 0.5 hr of incubation in 50% seawater ($P < 0.01$) and continued to be elevated at 1 hr of incubation ($P < 0.01$). Exposure to 150% seawater resulted in a significant induction of abdominal muscle HSP70 mRNA levels over control levels at 1 hr ($P < 0.01$).

HSP70 mRNA levels returned to control levels in both salinity exposure groups by 2 hr. Both hypo- and hyper-osmotic stress significantly induced HSP90 mRNA levels in lobster abdominal muscle at all time points examined (Fig. 6B). In 50% seawater, HSP90 mRNA levels were significantly greater than control levels by 0.5 hr of exposure ($P < 0.001$). HSP90 mRNA levels also remained significantly elevated at the 1 hr ($P < 0.001$) and 2 hr ($P < 0.01$) time points of the hypo-osmotic treatment. In 150% seawater, abdominal muscle HSP90 mRNA levels were significantly increased over control levels by 0.5 hr of exposure ($P < 0.001$) and remained elevated at the 1 hr ($P < 0.01$) and 2 hr ($P < 0.01$) time points.

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Premolt versus intermolt claw muscle (*P* < 0.01; Fig. 7A). There were no significant differences between intermolt and premolt HSP70 mRNA levels in claw muscle (Fig. 7A).

There were no significant differences in either HSP70 or 90 expression between premolt versus intermolt abdominal muscle (Fig. 7B). Comparison of premolt claw and abdominal muscle gene expression profiles revealed significant differences between the two tissues. HSP90 mRNA levels were significantly higher in premolt claw versus premolt abdominal muscle (*P* < 0.01; Spees *et al.*, 2003). However, HSP70 mRNA levels were not significantly different between tissues.

**Discussion**

We described an ELISA that proved sensitive enough to monitor levels of CHH by repeated sampling of small volumes of hemolymph from the same animal. In our juvenile lobsters, the handling and sampling per se did not significantly stimulate CHH release into the hemolymph. Therefore, the influence of some environmental stresses could be studied without significant interference from this handling stress. Another advantage of the ELISA is that hemolymph samples can be assayed directly, without extraction or hormone enrichment procedures.

We have assayed animals that were subjected to three stresses: emersion (producing hypoxia), temperature elevation, and salinity changes. Our results are in agreement with those of Webster (1996) from *Cancer pagurus*. He found that emersion causes a significant increase of CHH in the hemolymph after 15 min. Similarly, in our lobsters, a significant increase was measurable after 20 min. Webster (1996) discussed the physiological significance of this mechanism of endocrine metabolic adaptation for *C. pagurus*, which may be repeatedly subjected to short-term emersion and hypoxia in the intertidal zone. Lobsters may also occasionally experience hypoxia in warm, intertidal waters (Lawton and Lavalli, 1995). The increase of CHH in response to thermal stress may be related to either the hypoxic conditions existing in seawater and resulting from elevated temperatures, or to increased general metabolism at higher temperatures. Our results are consistent with observations made on thermal stress on crabs by Chung and Webster (1996).

Lobsters are considered to be stenohaline (Lawton and Lavalli, 1995). However *H. americanus* occasionally experiences hyposaline environments and can survive at salinities as low as 9 ppt (McLeese, 1956) and may be exposed to salinities as low as 0 ppt during winter snow run-off (reviewed in Charmantier *et al.*, 2001). The limited ability to osmoregulate would appear to be consistent with the limited metabolic adaptation to salinity changes. The observation that CHH increases only slightly upon salinity stress may reflect this situation.

In Norway lobsters patently infected with *Hemato-dinium* sp., the plasma CHH concentration shows a steady and significant increase in relation to infection severity (Stentiford *et al.*, 2001). As the parasite burden increases, a steadily increasing demand is placed upon the hosts’ hemolymph glucose. A feedback loop likely results in the release of additional CHH from the sinus gland. The parasites could also diminish the partial pressure of oxygen in the hemolymph via a reduction in hemocyanin. Thus, the elevated hemolymph CHH concentration in patent infection may be due primarily to a chronic “functional hypoxia” in the infected lobster, which elicits a cascade response similar to that seen during the “environmental hypoxia” caused by emersion. There is evidence that other stresses, such as exposure to heavy metals (Reddy *et al.*, 1996), capture in a towed trawl (Chang *et al.*, 2005), and contact with pesticides (De Guise, Maratea,
Chang, and Perkins, unpublished) cause increased secretion of CHH. These latter stresses (parasitic infection and exposure to toxins) are relatively long-term challenges (on the order of weeks in duration). We have not yet examined the effects of chronic environmental stresses (hypoxia and thermal and salinity acclimatization) on CHH.

Following heat-shock, protein immunoblotting results revealed an increase in HSPs in juvenile abdominal muscle, whole embryos, and whole larvae. Due to their more rapid and quantitatively greater responses, we focused our subsequent experiments on HSP mRNA expression. We used homologous molecular probes to lobster HSPs to quantify *H. americanus* gene expression. Data from the scans were normalized against the actin signal (data not shown) and expressed as percentage of control (100% seawater) mRNA level (n = 4 for all time points). Error bars represent one SD of the mean. Significance between treatment and control mRNA levels is indicated by the asterisks ($P < 0.01$, **; $P < 0.001$, ***). Modified from Spees et al. (2002a).
expression in vivo in different tissues and over short-term recovery periods (Spees et al., 2002b). The acute heat stress of 26°C used in our studies is in the range of the higher temperatures experienced by intertidal juveniles in nature (Reynolds and Casterlin, 1979; Lawton and Lavalli, 1995).

Current work in our laboratory is correlating HSP induction and induced thermotolerance. Induced thermotolerance is the ability of an organism to survive a usually lethal elevation in temperature if it is initially exposed to a brief sub-lethal temperature elevation. We have not yet studied long-term effects of elevated temperatures in any great detail. Lobsters can survive long-term acclimation to 30°C, a temperature close to lethality (∼32°C) (McLeese, 1956; Reynolds and Casterlin, 1979). In nature, lobsters are most likely to experience large temperature changes over seasons, or during on-shore summer migrations into coves and estuaries.

Other eurythermal ectothermic marine organisms have been examined for their HSP responses. Oysters (Crassostrea gigas; Clegg et al., 1998; Hamdoun et al., 2003), snails (Tegula spp.; Tomanek and Somero, 1999, 2002), limpets (Collisella spp.; Sanders et al., 1991), and teleost fish (Gillichthys mirabilis; Dietz, 1994), are all able to synthesize and accumulate HSPs following acute thermal stresses of magnitudes similar to those used in our studies.

Our results demonstrated that exposure to 50% or 150% seawater significantly induced lobster HSP gene expression. As described above in the discussion on CHH, lobsters often encounter hyposaline water but rarely hypersaline conditions. Perhaps this accounts for the observations that hyposalinity had a greater effect upon HSP expression than did hypersalinity. The low availability of ions likely perturbs cells by affecting enzyme-ligand interactions rather than by altering protein conformations (Somero and Yancey, 1997). Lobsters are poor osmoregulators. Their hemolymph osmolarity is significantly altered within 30 min of being placed in either hypo- or hypersomotic water (Spees et al., 2002a).

We observed tissue specific differences in HSP expression. For example, abdominal muscle displayed a significant increase in HSP90 mRNA after 2 hr of heat-shock, whereas an increase was seen in the hepatopancreas only after 2 hr of heat-shock followed by 6 hr of recovery at ambient temperature (Spees et al., 2002b). Hyposaline conditions increased HSP90 expression in abdominal muscle, but not in the hepatopancreas (Spees et al., 2002a). Thus, not all tissues respond in concert to a given stress. These limited observations imply that the HSP response in abdominal muscle is more sensitive to thermal and osmotic stress than the hepatopancreas. Supporting these data is the observation that after heat-shock the hepatopancreas (and not abdominal muscle) showed significant expression of the polyubiquitin gene. This is an indicator of increased protein degradation (Spees et al., 2002b) or “irreversible” damage (Mykles, 1998). This implies that abdominal muscle may have been more stable than hepatopancreas over the thermal interval tested. The protein pools that make up these tissues are thus likely to differ in their stability characteristics.

We found significant in vivo differences in HSP90 mRNA levels for lobster claw and abdominal muscle types at different molt stages. Fundamental physiological changes required for molting such as premolt-driven claw muscle atrophy (Mykles, 1992) are likely to account for the differences we observed. Molt cycle-dependent muscle atrophy is a novel example of HSP90 mRNA induction in a differentiated somatic tissue that is not undergoing environmental stress. This may relate to the role of HSP90 in multiple signal transduction pathways. For example, HSP90 transcription in H. americanus is influenced by the molt-cycle (it is elevated in premolt) and in response to ecdysteroids (Chang et al., 1999; Spees et al., 2003; Spees, unpublished data). In Drosophila, the ecdysteroid receptor is partially activated by HSP90 (Arbeitman and Hogness, 2000) as are most steroid receptors studied to date (Pratt, 1997).

I believe that our results presented in this paper demonstrate that measurements of hemolymph CHH and cellular HSPs will be useful for monitoring a variety of stress responses in lobsters. Crustaceans are keystone species in aquatic ecosystems and our studies should have utility in the monitoring of ecosystem
health and in improving fisheries and aquaculture practices. In addition, these studies point to avenues of further research into the economic regulation and signal transduction pathways of crustaceans. We observed that lobsters responded to some acute stresses only briefly (for example, the CHH response to acute thermal stress). Long-term studies will be required to determine the utility of this work for such chronic stresses as environmental pollution and global warming.

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REFERENCES


