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ORIGINAL RESEARCH

Hsp70 and Hsp90 are differentially expressed in crayfish muscle and neurons after heat stress

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Abstract: Heat shock proteins are essential cellular proteins that are highly conserved across organisms and contribute to adaptive responses of organisms during changing environmental conditions. Protein members of the families of heat shock genes can be differentially regulated in response to stressors and play critical roles in protein stability, folding, and molecular trafficking. We used a crustacean species with strong adaptability to diverse environments, the crayfish *Procambarus clarkii*, to study expression profiles of two well known heat shock genes, *Hsp90* and *Hsp70*. This crayfish can withstand a broad range of temperatures, and its adaptability contributes to its value for human use as an agricultural food source and as a biological control agent against snails that transmit schistosomiasis. However, it has become a harmful invasive species in some areas. To begin to understand the thermal resilience of *P. clarkii*, we identified and cloned *Hsp90* from crayfish by degenerate polymerase chain reaction in conjunction with rapid amplification of 3′ and 5′ cDNA ends, and subsequently sequenced and characterized the molecular chaperone. Sequence analysis by phylogenetic alignment and polypeptide three-dimensional structure prediction of the newly identified *Hsp90* gene shows that it has conserved motifs with *Hsp90* s in other species. Using quantitative polymerase chain reaction, we characterized the expression profiles of *Hsp90* and *Hsp70* in muscle and in central nervous system tissues. We found that *Hsp70* and *Hsp90* transcripts are upregulated under heat stress in both muscle and the central nervous system, but that their expression levels are more robustly increased in muscle.

Keywords: crayfish, stress response, *Procambarus clarkii*, heat shock protein, Hsp90, schistosomiasis

Introduction

Procambarus clarkii is a freshwater crayfish species native to southern North America, with a high tolerance to environmental extremes.[1] When compared with another closely related crayfish species, *P. clarkii* has a much greater survival rate after exposure to photoperiod stresses[2] and to high temperatures.[3] *P. clarkii* has a temperature preference of around 22°C, but can acclimate successfully to temperatures from 5°C to 35°C.[1] The ability of the American crayfish to adapt to diverse conditions has led to its success as an invasive species in many areas of the world, particularly in Europe and Africa, and to its use as one of the most important freshwater decapods farmed for human consumption.[4] Since its introduction in Africa in the mid 1900s, it has also had a significant impact on reduction of the snail populations that serve as intermediate vectors[5–7] for transmission of the parasitic schistosome worms responsible for more than 200 million human infections annually.[8] The resilience of *P. clarkii* makes

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it an interesting model for the study of cellular tolerance to environmental changes, such as temperature extremes.

Heat shock proteins are a family of evolutionary conserved proteins that mediate the homeostatic cellular response to stress,[9] and were first characterized in *Drosophila* after exposure to temperature extremes.[10–14] Transcription of genes encoding heat shock proteins can be induced by a variety of stressors, including osmotic changes, hypoxia, exposure to toxins, infections, a range of pathologic conditions, and protein damage.[9–11] Heat shock proteins primarily function as molecular chaperones, facilitating protein synthesis and folding, and maintaining structural integrity while participating in the regulation of transcription factors and protein kinases in order to assist in the modulation of signal transduction cascades and sustain cellular homeostasis.[15,16]

Heat shock proteins are grouped into six major families defined according to their molecular weight, and of these, Hsp70 and Hsp90 are the most abundant.[9] The Hsp70 family of heat shock proteins represents one of the largest stress families distributed across organisms. Hsp70 prevents proteins from aggregating by binding tightly to partially synthesized polypeptides, assists in the transmembrane transport of proteins, and participates in the disposal of damaged or defective proteins.[17–19]

Hsp90 is the most abundant of the heat shock proteins, representing almost 1% of the total cellular protein in unstressed cells.[10,20] It functions as a molecular chaperone in maturation and activation of the proteins that are important for growth and development, such as members of the steroid receptor family,[21–23] several protein kinases (Src family, Raf family, MAP kinases),[22–25] the tumor suppressor p53,[26] and telomerase.[25,27] Under stress conditions, Hsp90 also works as a protective agent in protein renaturation and refolding.[28]

Both Hsp70 and Hsp90 play a role in neuronal development and cellular maintenance, but can also promote disease pathology.[29,30–36] For example, Hsp70 can act to protect the nervous system from toxic effects due to progression of neurodegenerative disease in mammals,[37] whereas Hsp90 is thought to maintain disease conditions in tauopathies. Hsp90 can also act to repress Hsp70 and other heat shock proteins.[38,39] Thus, both positive and negative transcriptional regulation of heat shock proteins is likely to be important for protection of the central nervous system (CNS) and other tissues. The relative expression of *Hsp70* and *Hsp90* genes is dependent on tissue specificity and stress type.

In studies examining the stress response in crustaceans, *Hsp70* and *Hsp90* are the most widely studied. As expected, expression of these genes is altered in response to thermal or other environmental stresses,[1,2,40–42] and for spermatogenesis.[43] In the CNS, these proteins may be required for axonal regeneration after molting[44] and as part of the unusual and extensive ability of crustaceans to recover from wounds and to repair damaged nervous tissue.[41,45,46] *P. clarkii* axons in particular are unusually resilient to insult and remain functional for months after injury.[41] Hsp70 and Hsp90 levels are elevated at sites adjacent to injured tissue.[47] In lobsters, Hsp70 protein is elevated after heat shock at 26°C for 2 hours, ie, 13°C above normal equilibrium temperature.[40] To date, only expression of Hsp70 has been reported in the *P. clarkii* CNS,[41,48] but nothing is known about Hsp90 expression in *P. clarkii*.

P. clarkii is one of the most successful invasive species of crayfish worldwide. Native to northeastern Mexico and the southern US, *P. clarkii* has adapted to a wide environmental global range on every continent except Australia and Antarctica.[49] Since Hsp70 and Hsp90 play important roles as stress proteins, we characterized the effects of thermal stress on *P. clarkii* by measuring transcript levels of *Hsp90* and *Hsp70* in claw muscle and the CNS in crayfish. In this study, we identified, isolated, cloned, and characterized full-length cDNA of the *Hsp90* gene from *P. clarkii*. Our simple hypothesis was that the transcript levels of *Hsp70* and *Hsp90* in crayfish would be elevated. Using quantitative real-time polymerase chain reaction (PCR), we show that *Hsp70* and *Hsp90* transcripts are both upregulated after thermal stress in *P. clarkii*, but also that these transcripts show significant differential transcript level patterns in the CNS and in muscle.

Materials and methods

Animals

Adult *P. clarkii* were obtained from Niles Biological Inc (Sacramento, CA, USA) and maintained on a 12-hour light-dark cycle in aquaria with water. Only intermolt animals were selected for the study.[50] The animals were acclimated to 20°C in individual tanks for at least one week prior to experiments to minimize nonspecific stress. The animals were fed pieces of carrot every 2 days.

Thermal stress

All experimental animals were exposed to acute thermal stress (35°C) for 2 hours in individual tanks as previously described.[51] Before treatment, a heater (CA200, Teco, Ravenna, Italy) was placed into the tank to increase the temperature from 20°C to 35°C, a 15°C thermal stress change. Five animals were sacrificed immediately on completion of the 2-hour thermal stress treatment. Portions of the claw

muscles, ventral nerve cord, and brain of each animal were removed and desheathed in a chilled crayfish saline solution (5.4 mM KCl, 205.3 mM NaCl, 13.5 mM $CaCl_2$, 2.6 mM $MgCl_2$, 2.3 mM $NaHCO_3$, and 2.0 mM dextrose, pH 7.2–7.4). For all nervous tissue described, samples are a combination of brain and ventral nerve cord. Five unstressed control animals were sacrificed and tissue samples were collected as described above.

Cloning of *Hsp90* gene

We cloned and sequenced the complete Hsp90 from the *P. clarkii* crayfish. First, 3′ rapid amplification of cDNA ends (RACE) using degenerate primers was based on conserved amino acid and nucleotide alignments from *Portunus trituberculatus*, *Eriocheir sinensis*, *Chiromantes haematocheir*, *Penaeus monodon*, *Drosophila melanogaster*, and *Homo sapiens*. The primer for 5′ RACE was designed based on DNA sequences identified by 3′ RACE.

Total RNA was extracted from muscle and CNS tissues of the experimental and control animals using the PureLink RNA mini kit with TriZol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DNAseI was performed to eliminate DNA contamination. Both 3′ and 5′ RACE were performed with the SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA). Briefly, for 3′ RACE, first-strand cDNA was reverse-transcribed from 1 µg total RNA using the 3′ RACE cDNA synthesis primer (3′-CDS) from the kit. Next, a partial sequence of the *P. clarkii Hsp90* transcript was selectively amplified using a degenerate *Hsp90* primer oLS090 (5′-CARTTYATTGGCTAYCCMATCAAG-3′) and the universal primer mix. PCR was performed by incubation of cDNA with Phusion polymerase in a Multigene gradient (Labnet, Edson, NJ, USA) programmed for 32 cycles of the following temperature schedule: 98°C for 8 seconds, 61°C for 20 seconds, and 72°C for 20 seconds. For 5′ RACE, first-strand cDNA was reverse-transcribed from 1 µg of total RNA using 5′-CDS and SMART-IIA oligonucleotide provided in the kit. In the following PCR step, selective amplification of 5′ *Hsp90* sequences used the primer oLS091 (5′-CCACGTTCGGCATGTCGTCTTTTTTAGCAGCCTCGT-3′) based on 3′ RACE sequencing and a universal primer mix. PCR was performed as described earlier. After characterization on agarose gel and purification with the Wizard® SV gel and a PCR clean-up system (Promega, Madison, WI, USA), 3′ and 5′ RACE PCR products were cloned into a PCR 2.1 vector (Invitrogen) for transformation of TOP10 chemically competent *Escherichia coli* cells (Invitrogen) and sequenced by Elim Biopharmaceuticals Inc (Hayward, CA, USA) to identify the complete cDNA sequence and predict the protein sequence (Figure 1). The *P. clarkii Hsp90* transcript sequence was submitted to Genbank and given the accession number JQ995601.

Quantitative PCR

Primers specific for quantitative PCR detection of *Hsp70* (Genbank, DQ301506.1), *Hsp90*, and β-actin (Genbank, FJ389458.1) were designed using Primer3 software (http://frodo.wi.mit.edu/). The primers are listed in Table 1. The cDNA was prepared using a high-capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's guidelines. First, 150 ng cDNA was used as a template in a 60 µL PCR reaction using Power SYBR Green Master Mix (Applied Biosystems) with 900 nM of the forward and reverse primers. Each reaction was split in 20 µL triplicates and PCR was performed on a StepOnePlus™ real-time PCR system (Applied Biosystems). The samples were run for 40 cycles using the following program: 94°C for 15 seconds, 60°C for 30 seconds, and 72°C for 30 seconds. Detection of SYBR Green fluorescent intensity occurred at 72°C of each cycle and was analyzed by StepOne system software. *β-actin* was used as the reference gene in all comparative threshold cycle ($\Delta\Delta C_T$) experiments.[52–54] The melt curve for each pair of primers revealed only one PCR product for any given reaction.

Sequence analysis

The translated open reading frame of *P. clarkii Hsp90* cDNA was determined using a publicly available translation tool (http://web.expasy.org/translate/). The molecular mass and theoretical isoelectric point was calculated using the pI/Mw

Table 1 Gene names and DNA primer sequences used for quantitative real-time polymerase chain reaction analysis

Gene name	Forward primer	Reverse primer	Amplicon size
Primers for qRT-PCR			
Hsp70	CGAGAGAGCCAAACGAACTC	CAACACCAGTTCATGGATG	243 base pairs
Hsp90	AAAAAGACGACATGCCGAAC	AGTGGTCCTCCCAGTCATTG	219 base pairs
β-actin	CTGAGCGTGGCTATTCCTTC	AAGGAAGGCTGGAAGAGAGC	175 base pairs

Abbreviations: *Hsp, heat shock protein;* qRT-PCR, quantitative real-time polymerase chain reaction analysis.

tool (http://web.expasy.org/compute_pi/). The Simple Modular Architecture Research Tool (SMART) program (http://smart.embl-heidelberg.de/) was used for motif predictions. DNA and amino acid similarity searches were conducted using Basic Local Alignment Tool for nucleotides (BLASTn) or proteins (BLASTp) with nucleotide or protein collection databases (http://blast.ncbi.nlm.nih.gov/). Deduced amino acid sequences were aligned with other Hsp90 proteins from arthropod species and a phylogenetic tree was generated using the neighbor-joining method (http://www.ebi.ac.uk/Tools/msa/clustalw2/). Two chelicerates, *Opistophthalmus carinatus* and *Tetranychus cinnabarinus*, were used as outgroup controls and the gap penalty of the phylogenetic tree was set as the default value (Figure 2). The three-dimensional protein structure was predicted using the I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/).[55,56]

Results

Identification and cloning of *P. clarkii* Hsp90

The *P. clarkii Hsp90* gene was identified and cloned based on sequence homology with previously reported Hsp90 proteins from other decapod crustacean species, ie, *P. trituberculatus*, *E. sinensis*, *C. haematocheir*, and *P. monodon*, an insect *D. melanogaster*, and *Homo sapiens*. A 2.63 kb cDNA clone encoding the full-length crayfish *P. clarkii Hsp90* transcript was cloned by 5′ and 3′ RACE. It included an 82 base pair 5′-terminal untranslated region, a 383 base-pair 3′-terminal untranslated region with a canonical polyadenylation signal sequence AATAA, and a 2,167 base pair open reading frame encoding a 718-amino acid protein. *P. clarkii* Hsp90 protein has a predicted molecular mass of 82.8 kDa and a theoretical isoelectric point of 4.88. The protein sequence contains five conserved amino acid motifs (Figure 1I–V) regarded as Hsp90 protein family signatures that are used to identify this family of proteins.[57] In eukaryotes, Hsp90 has been found in the endoplasmic reticulum and in the cytosol. Cytosolic Hsp90 proteins have the conserved MEEVD sequence in their C-terminal ends, and a similar sequence (V/I) EEVD is seen in Hsp70 proteins. We observed the presence of a C-terminal sequence MEEVD motif which indicates that crayfish *P. clarkii* Hsp90 is a member of the cytosolic Hsp90 family (Figure 1, open box).[57]

Phylogenetic analysis of *P. clarkii* Hsp90

To further characterize *P. clarkii* Hsp90 protein relative to other arthropod Hsp90 proteins, we constructed an Hsp90 phylogenetic tree using ClustalWII (Figure 2). Phylogenetic analysis of the Hsp90 proteins sorted the tested samples into two groups made up of Crustacea and

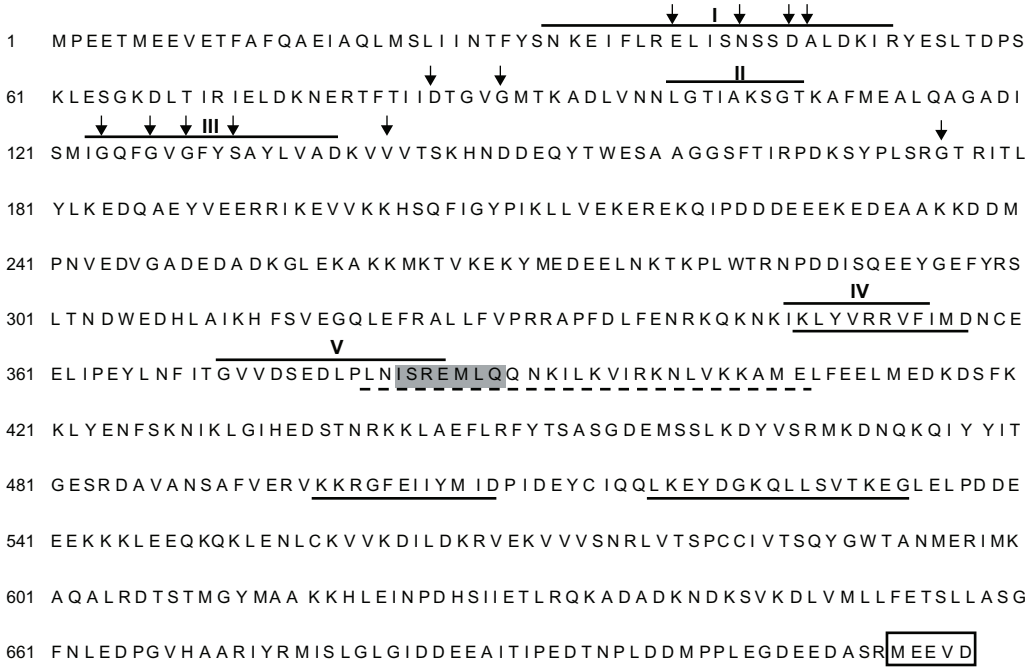

Figure 1 Amino acid sequence deduced for *Procambarus clarkii* heat shock protein 90. The numbers at the left refer to the amino acid residue positions. Heat shock protein 90 (Hsp90) signature sequences are indicated by the overline and roman numerals I–V. An open box indicates the cytosolic Hsp90 signature MEEVD. The putative leucine zipper is indicated by a dashed underline. The IRExLQ motif is highlighted in gray. Three putative adenosine triphosphate binding sequences are indicated by underline. Arrows indicate the amino acids involved in nucleotide and geldanamycin binding.

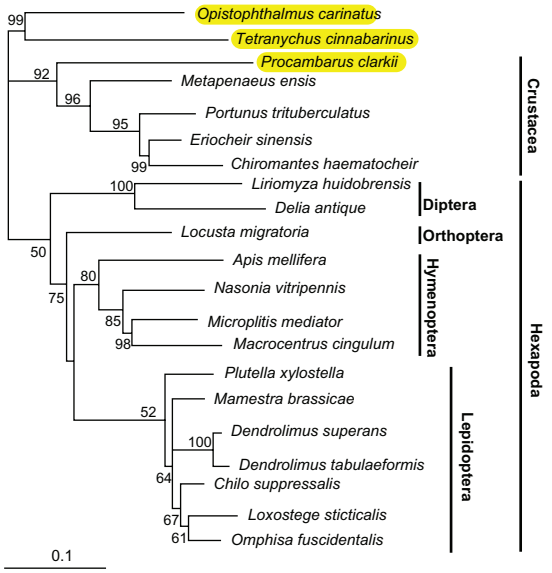

Figure 2 A phylogenetic tree of heat shock protein 90 family members constructed based on the amino acid sequence of 21 *heat shock protein 90* genes. The number at each branch indicates the percentage of times that a node was supported in 1,000 bootstrap replications by the neighbor-joining method. The scale bar represents 0.1 substitutions per site. The chelicerates are highlighted in yellow.

Hexapoda. Within Hexapoda, Diptera is basal to all other taxa, and Orthopera is a sister group to Lepidoptera and Hymenoptera. Within Crustacea, *P. clarkii* Hsp90 protein is closely related to the Hsp90 proteins from the Japanese blue crab (*Portunus trituberculatus*), Chinese mitten crab (*Engleromyces sinensis*), and a prawn (*Metapenaeus ensis*). However, the heat shock proteins from these decapod marine crustaceans are phylogenetically closer to each other than to the freshwater crayfish (Figure 2).

Structural analysis of *P. clarkii* Hsp90

The three-dimensional structure analysis prediction of *P. clarkii* Hsp90 revealed two distinguishable domains connected by a highly charged loop (Figure 3A). The N-terminal domain (C-score 1.89) consists of eight-stranded parallel β-sheets and nine α-helices (Figure 3B), which is highly similar to Hsp90 proteins in other species.[58,59] The C-score estimates the accuracy of the structure predictions and typically ranges from −5 to 2, with a higher score correlating with higher model confidence.[55] The three-dimensional structure is consistent with its functional amino acid sequence (arrowed amino acids in Figure 1), indicating its conserved function for nucleotides, geldanamycin. and other substrate target protein binding. The middle domain (C-score 1.48) contains eleven-stranded β-sheets separated by α-helices (Figure 3C), revealing the conservative adenosine triphosphate (ATP) binding dock. The three-dimensional structure prediction C-score is significantly higher than its cutoff value, ie, −1.5, suggesting that the simulated images are reliable.

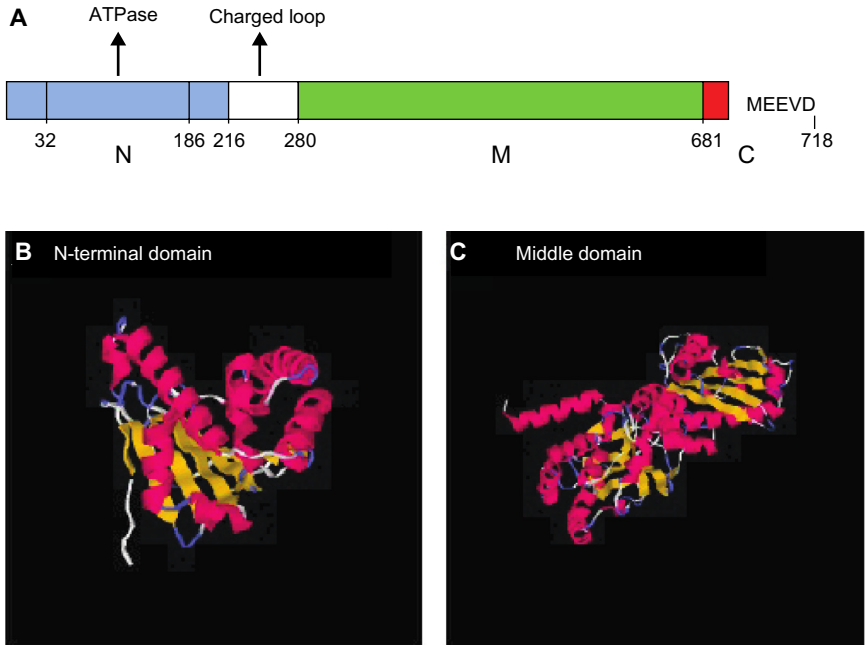

Figure 3 Predicted structure of *Procambarus clarkii* heat shock protein 90. (**A**) Graphic description of *P. clarkii* heat shock protein 90 domains. Amino acid numbers for domain boundaries are labeled. N-terminal, middle, and C-terminal domains are shown in blue, green, and red, respectively. ATPase (32–186) is shown in the N-terminal domain. (**B** and **C**) Three-dimensional structure of the N-terminal (**B**) and middle (**C**) domain, respectively; α-helices are shown in pink; β-sheets are shown in yellow; loops are shown in white with anion binding structures located in the chain (blue).
Abbreviation: ATPase, adenosine triphosphatase.

P. clarkii Hsp90 and *Hsp70* expression induced by thermal stress

Quantitative PCR was used to determine whether the expression level of heat shock proteins varies in muscle and nerve tissue under thermal stress conditions. While the β-actin internal control was constitutively and comparably expressed in tissues from control animals and animals exposed to thermal stress, both muscle and nerve tissue showed variability of *Hsp90* and *Hsp70* transcript levels under stress conditions (Figure 4). After thermal stress, *Hsp70* and *Hsp90* transcript levels were significantly upregulated (Figure 4). *Hsp90* transcript levels increased 18.1-fold in muscle tissue and 3.4-fold in nerve tissue. For *Hsp70*, transcript levels increased 9.4-fold in muscle and 3.6-fold in nerve. For both *Hsp90* and *Hsp70*, the transcript level was elevated in both nerve and muscle, but mainly in muscle. Within muscle, the *Hsp90* transcript level was more elevated (18.1-fold) than the *Hsp70* transcript in muscle (9.4-fold).

Discussion

We have identified, cloned, and characterized *Hsp90* from the American crayfish *P. clarkii*. Our data demonstrate that *Hsp70* and *Hsp90* transcripts are both upregulated in response to heat stress in crayfish as expected, but that the transcriptional response of the heat shock proteins to thermal stress in muscle and in the CNS is distinctly different. Using quantitative real-time PCR, we observed that *P. clarkii Hsp70* and *Hsp90* transcripts are both upregulated by 9.4-fold and 18.1-fold, respectively, in muscle, whereas this elevation was only around 3.5-fold in neurons for both transcripts. In the American lobster, *Homarus americanus*, abdominal muscle is more stable in the 2 hours following heat stress when compared with the hepatopancreas,[44] suggesting that in some cases muscle tissue may be more resilient to stress than other tissues, which correlates with our observations of elevated heat shock proteins after stress. Thus, a greater increase in relative gene expression in muscle seems consistent with greater protection, and therefore better adaptation to environmental stress.[60,61] Consistent with results from other systems, our data show induction of RNA transcript levels in response to temperature stress, in accordance with the roles of Hsp90 and Hsp70 in cellular homeostasis in *P. clarkii*.

Several structural factors led us to conclude that we have identified and cloned *P. clarkii Hsp90*. Eukaryotic Hsp90 proteins have functionally conserved N-termini and C-termini connected by a middle region containing highly charged and hydrophobic regions with variable length.[24,62] The N-terminal domain is an α/β sandwich composed of an eight-stranded antiparallel β-sheets and nine α-helices,[24,63] containing an ATP binding and a peptide binding site.[24,58,63] The middle domain of Hsp90 has a "client" protein-binding site and part of a "split ATPase".[18,64] The C-terminus has an alternative ATP binding site and "client" protein-binding site.[24,62] Our analysis of Hsp90 suggests that the N-terminal

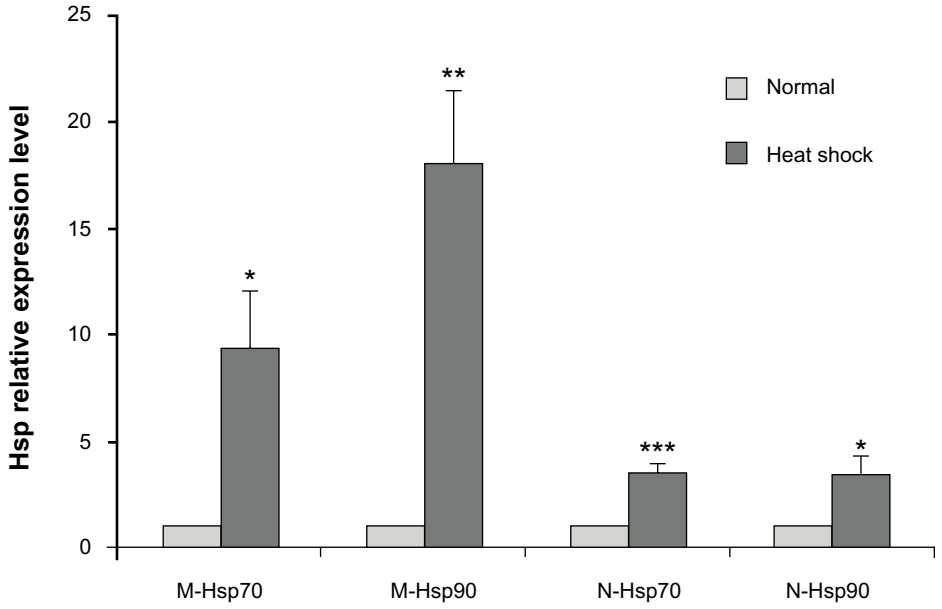

Figure 4 Hsp70 and Hsp90 relative mRNA expression levels ($2^{-\Delta\Delta Ct}$) in muscle and CNS tissue under normal and heat shock conditions. For each bar, n=3, the error bars represent one standard deviation of the mean. Asterisks indicate a significant difference between treatment and the respective control (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$).
Abbreviations: CNS, central nervous system; Hsp, heat shock protein; M-Hsp70, Hsp70 expression in muscle; M-Hsp90, Hsp90 expression in muscle; N-Hsp70, Hsp70 expression in the CNS; N-Hsp90, Hsp90 expression in CNS mRNA, messenger RNA.

region contains amino acid residues related to the binding of ATP/ADP, or cochaperone p23, a phosphoprotein that stabilizes the interaction between Hsp90 and interacting client proteins (Figure 1, arrows).[42,65–67] These residues can constitute a pocket-like structure unique to Hsp90, called the "Bergerat fold", which is an α/β sandwich with high affinity for ATP.[68] This nucleotide-binding site can also be bound by the antibiotic geldanamycin or similar ansamycin antibodies that, in turn, prevent p23 binding and block Hsp90 function.[69,70] We used the SMART program version 7 that verified a histidine kinase-like ATPase domain spanning amino acids 32–186 (Figure 3A), and an Hsp90 protein family domain spanning amino acids 188–718, common in Hsp90 family members.[71,72] The middle region of *P. clarkii* Hsp90 contains part of the split ATPase of Hsp90 proteins (motif ISRExLQ, Figure 1, gray),[18] and putative leucine repeats with motif $LX_6\ LX_5\ LX_6\ LX_6$ (Figure 1, dashed underline), which is consistent with a client protein binding site in this middle region, as reported previously.[18] This region also contains three putative ATP binding sequences (Figure 1, underline).[20] At the C-terminal end of the amino acid sequence is a tetratricopeptide repeat motif recognition site, ie, the conserved MEEVD pentapeptide, which is responsible for interaction with cochaperones such as immunophilins FKBP51, stress-induced phosphoprotein 1, and others.[59] Detection of these characteristic Hsp90 structural and functional domains in the crayfish *P. clarkii* Hsp90 amino acid sequence identifies it as a functional member of the Hsp90 family.

As in other crustacean species, including the lobster,[40] blue crab,[42] and mitten crab,[73] *Hsp70* and *Hsp90* are significantly induced by thermal stress in the muscle tissue of crayfish (Figure 4). However, relative to *Hsp70*, *Hsp90* transcript levels are higher. Greater accumulation of *Hsp90* transcripts under thermal stress might suggest a significant role in protection against cellular damage. Alternatively, our analysis using comparative quantitative real-time PCR could be due to a greater reserve of heat shock proteins in nervous tissue relative to muscle tissue, which could explain why the dramatic increase in relative transcript level is significantly lower in nervous tissue.

The CNS is also sensitive to thermal stress,[74] and the heat shock response in many types of neurons may contribute to this process.[75–78] Our quantitative real-time PCR results show that both *Hsp70* and *Hsp90* exhibit significantly more upregulation after thermal stress in muscle than in the nervous system (Figure 4). There are several explanations for this observation. First, the pathway of regulation for heat shock proteins in the nervous system varies from that in muscle. Previous studies show that motor neurons have a high threshold for inducing a heat shock response,[79,80] which is attributed to an impaired ability to activate heat shock transcription factor 1.[75] In addition, Taylor et al[81] showed that the heat shock response of Hsp70 in motor neurons of the embryonic mouse spinal cord was not due to phosphorylation of heat shock transcription factor 1, a requirement for transactivation of heat shock genes in non-neuronal cells.[82–84] It is due to a calcium/calmodulin-dependent kinase, suggesting different mechanisms of activation of Hsp70 in motor neurons and non-neuronal cells. The same group also showed that the protection of motor neurons partly depended on circulating extracellular heat shock proteins, suggesting that stores of *Hsp70* and *Hsp90* could contribute to the thermal stress response in the nervous system. They hypothesized that motor neurons synthesize only the amounts of Hsp70 necessary for maintenance of cell function and survival, but do not increase production in response to the greater demands of environmental stress. Although neurons can be protected by heat shock proteins from surrounding cells, upregulation of endogenous heat shock proteins in neurons (Figure 4, Hsp70 increases 3.6-fold and Hsp90 increases 3.4-fold in the CNS) may provide efficient protection from stress. Therefore, to further determine the role of heat shock proteins in neuronal protection, measuring the expression level of heat shock proteins in single neurons of known type and function could be useful in future studies. Third, Hsp70 and Hsp90 are not the only protective chaperones playing a role in the heat shock response. The same study also showed that overexpression of constitutively active heat shock transcription factor 1 was better able to confer neuroprotection than Hsp70 alone.[85] Heat shock transcription factor 1 functions to activate heat shock genes, and the inability of Hsp70 to induce protection suggests that there may be other factors involved in the heat shock response.[85] Upregulation of other chaperones, in particular Hsp40, are more efficient than Hsp70 and Hsp90 alone for neuroprotection in mouse primary cells of dissociated spinal cord in reducing toxicity and preventing aggregation of misfolded proteins induced by thermal stress.[85]

Conclusion

In this study, we cloned, sequenced, and identified *Hsp90* from crayfish. Our results show that the transcript levels of *Hsp70* and *Hsp90* are elevated in response to thermal stress in the nervous system and muscle of crayfish, but that this elevated transcriptional response occurs at differential levels, being significantly greater in muscle cells. These data corroborate a function for Hsp70 and Hsp90 as stress-sensing heat shock chaperones in crayfish. The increase in *Hsp70*

and *Hsp90* levels may also provide clues as to why *P. clarkii* is so adaptable in extreme environments. It may be useful to examine selective Hsp70 and Hsp90 inhibitors to investigate the mechanism governing gene expression of heat shock proteins in more crayfish tissues. In addition, it will be of interest to identify and clone other heat shock proteins in *P. clarkii*, and to determine if expression of heat shock proteins is distinct from that in less resilient crayfish species.

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Disclosure

The authors report no conflicts of interest in this work.

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