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Functional evaluation of Heat Shock Proteins 70 (HSP70/HSC70) on *Rhodnius prolixus* (Hemiptera, Reduviidae) physiological responses associated with feeding and starvation

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ABSTRACT

Blood-sucking vectors must overcome thermal stress caused by intake of proportionally large amounts of warm blood from their hosts. In response to this, Heat Shock Proteins (HSPs) such as the widely studied HSP70 family (the inducible HSP70 and the cognate form HSC70, known for their role in preserving essential cellular functions) are rapidly up-regulated in their tissues. The triatomine *Rhodnius prolixus* is an important vector of *Trypanosoma cruzi*, the causative pathogen of Chagas' disease, and is also a model organism for studying insect biology and physiology. In this work, we observed that the expression of *Rhodnius prolixus* HSP70 was rapidly up-regulated in response to thermal shocks (0 °C and 40 °C) and also during the first hours after feeding on blood. HSP70/HSC70 RNAi knockdown elicited important alterations in *R. prolixus* physiological responses triggered by blood meal and starvation. HSP70/HSC70 knockdown insects showed lower resistance to prolonged starvation in comparison to appropriate controls, dying between 32 and 40 days after dsRNA injection. After blood feeding, the physiological effects of HSP70/HSC70 knockdown were more prominent and the insects died even earlier, within 14–20 days after feeding (21–27 days after dsRNA injection). These bugs showed impaired blood processing and digestion, reduced energetic metabolism and the midgut immune responses were compromised. Our findings suggest that HSP70/HSC70 depletion affected *R. prolixus* in starvation or fed conditions. After feeding, the arrival of blood in the digestive tract of knockdown insects fails to activate essential signaling pathways involved in blood processing, producing several alterations in their physiological processes enough to generate a premature death.

1. Introduction

Temperature is a critical factor that can affect several parameters in the biology and physiology of insects. High temperatures induce alterations in their metabolism, respiration patterns and can change the functioning of insect's nervous and endocrine systems (Neven, 2000). An interesting example is reported for newly fed nymphs of *Rhodnius prolixus*. When these insects were kept at temperatures of 35 °C they were unable to perform ecdysis (Okasha, 1964). Therefore, some insect species have developed behavioural and physiological thermoregulatory strategies to counteract the stress caused by environmental heat exposure (Heinrich, 1993). It has been shown that, in order to cope with the thermal stress associated with feeding, they either activate molecular reparatory measures (Benoit et al., 2009) or actively cool down (Lahondere and Lazzari, 2012).

Insects and many other organisms have developed an important genetic mechanism to respond to heat stress and increase heat tolerance: the rapid induction of expression of Heat Shock Proteins (HSPs). In addition to the thermal stress (heat and cold), HSPs are

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essential molecules involved in many biological processes, including the adaptive response to a wide range of environmental stresses such as exposure to toxic substances or contaminants, anoxia and desiccation (Mahroof et al., 2005; Tammariello et al., 1999). These proteins have an important role in protecting essential cellular functions, acting as chaperonins that help to maintain the functional conformation of proteins allowing their translocation across cell membranes, minimising protein aggregates and assisting the removal of denatured proteins (Mahroof et al., 2005).

HSPs are grouped in different protein families based on their sequence homology and molecular masses. Among them, members of the Heat shock protein 70 (*Hsp70*) family are highly conserved (Gross et al., 2009) and are probably the best characterised and most studied HSP family in insects (Luo et al., 2015; Neven, 2000). The expression of HSP70 is considered a good marker for inducible stress response in an organism, directly interacting with other HSPs (Bettencourt et al., 2008; Colinet et al., 2010). In addition to the inducible HSP70, the heat-shock cognate protein 70 (HSC70) is also a constitutive member of the HSP family. These two proteins have a high degree of sequence homology and co-purify with one another, but the cognate form HSC70 is normally expressed under normal conditions (Prentice et al., 2004; Shiota et al., 2010). Due to their high similarity, previous studies failed to analyse their roles separately (Kostal and Tollarova-Borovanska, 2009). In this study, the authors produced dsRNA to specifically knockdown the HSP70 of the hemipteran bug *Pyrrhocoris apterus* but also observed a reduction in HSC70 mRNA and protein levels.

The haematophagous insect *R. prolixus*, one of the main vectors of *T. cruzi,* which is the causative agent of Chagas' disease – a chronic and debilitating disease infecting 7–8 million people in Latin America (WHO, 2015), has been widely used as a classical model organism to study insect biology and physiology. Sequences encoding proteins of the "Heat Shock" type, including members of HSP70 family, have been recently identified in the genome and digestive tract transcriptome of this triatomine bug (Ribeiro et al., 2014). However, no HSP functional study has been developed to date. Although thermotolerance was evaluated in the triatomine bug *Panstrongylus megistus*, the HSPs specific role was not directly investigated (Garcia et al., 1999, 2001, 2003). Using the closely-related species *Triatoma infestans,* Kollien and Billingsley (2002) suggested a possible role for HSP70 in the resistance of this insect to long periods of starvation. Thus, considering *R. prolixus'* position as a classical model for insect studies and its relevance for a major public health disease, there is a remarkable lack of information on the response of this triatomine species to stress. The haematophagic behaviour is a critical moment for vectors, parasites and vertebrate hosts, because it is the moment in time when they all congregate, representing the opportunity for the parasite to be transmitted.

In this work, we investigated the role of HSP70 family (HSP70/HSC70) in the physiological responses elicited by *R. prolixus,* especially associated with feeding and starvation. To this end, we monitored the expression levels of HSP70 transcripts in insects exposed to extreme environmental temperatures and after a blood meal. In addition, we knocked down the expression of HSP70 in *R. prolixus* nymphs to evaluate the impact of HSP70 family mRNA depletion on various physiological parameters and insect survival rates. The expression "HSP70 knockdown" used herein corresponds to RNAi depletion of the HSP70 family members (concomitant knockdown of the inducible HSP70 and its cognate form -HSC70). Finally, we evaluated whether the expression of genes related to endoplasmic reticulum stress and immune-related responses was altered by HSP70 knockdown before (in starvation) and after the blood feeding.

2. Material and methods

2.1. Insects

R. prolixus (Honduras) colony was reared under controlled conditions of temperature (28 ± 2 °C) and relative humidity (65 ± 10%), 12/12 h light/dark cycle and insects were allowed to feed weekly on hamsters. Experimental insects (5th instar nymphs) were fed on anesthetised hamsters or heparinised sheep blood in an artificial feeder at 37 °C when monitored in a respirometer. The experiments were approved by the Ethical Committee on Animal Use (CETEA-UFMG) under the protocol number 115/2011.

2.2. RNA extraction and cDNA synthesis

RNA from midgut pools of *R. prolixus*' 5th instar nymphs was extracted using the Nucleospin RNA II Kit (Macherey-Nagel) and treated with DNase according to the manufacturer's instructions. The RNA was eluted in 20 μL of ultra-pure RNase-free water. We used mice keratin (NM_027574) gene sequence to produce the unrelated dsRNA used in control groups. RNA from mouse tail epithelium was extracted with Trizol reagent (Invitrogen) followed by treatment with Turbo DNA-free (Applied Biosystems). RNA was quantified by measuring 260 nm wavelength absorbance. RNA extraction quality/purity was assessed by the 260 nm/280 nm absorbance ratio. RNA (0.5 μg) was used for cDNA synthesis with 0.5 μg of random hexamers (Promega) using the M-MLV reverse transcriptase system (Promega) in 25 μL final volume. This method was used to produce cDNA for both PCR and quantitative PCR (qPCR).

2.3. PCR and dsRNA synthesis and delivery

One microlitre of the synthesised cDNA (10 ng) was used as a template for PCR amplification of the target genes. PCR was carried out using 200 nM of specific primers conjugated with 23 bases of the T7 RNA polymerase promoter at the 5′ end (taatacgactcactatagggaga). The PCR primers and amplicon sizes were: HSP70 forward 5′- T7 + ccaagcgagcattatcatca -3′ and reverse 5′- T7+ tccagcaatacaatcgcatc -3′ (416 bp amplicon), Keratin forward 5′- T7 + ggggtctcctctctggaaac-3′ and reverse 5′- T7 + attagcagccgtggaagaga-3′ (275 bp amplicon). A total of 35 cycles were carried out under the following PCR thermocycling conditions: denaturing step at 94 °C for 40 s; annealing step at 60 °C for 40 s and extension step at 72 °C for 45 s. All reactions contained 200 mM deoxyribonucleotide triphosphate (dNTP) and 1 unit of Taq DNA polymerase (Invitrogen) in 20 μL final volume. PCR products were used as template for dsRNA synthesis using the MegaScript T7 Transcription Kit (Ambion) according to the manufacturer's instructions. After synthesis, the dsRNA was isopropanol-precipitated, eluted in ultra pure water and quantified by 260 nm wavelength spectrophotometry. The quality of the dsRNA products was verified by 2% agarose gel electrophoresis. The dsRNA was air-dried, dissolved in sterile saline solution (NaCl 0.9%) at 10 μg/μL and 1 μL was injected into the thoracic haemolymph of each *R. prolixus* 5th instar nymph (under 10 days of starvation after molt) with a microinjector (Nanoinjector, Drummond, USA). The control group was injected with the same amount of keratin dsRNA.

2.4. Real-time quantitative polymerase chain reaction (qPCR)

The reactions were conducted using the StepOne Plus real time quantitative PCR system (Applied Biosystems) to evaluate the HSP70 expression under different experimental conditions, to access the reduction in mRNA levels after HSP70 knockdown and also evaluate the expression of genes related to unfolded protein