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Characterization of Hsp70 gene in *Chironomus riparius*: Expression in response to endocrine disrupting pollutants as a marker of ecotoxicological stress

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ABSTRACT

We characterized the Hsp70 cDNA in *Chironomus riparius* and evaluated its expression profile under different environmental stressors. It is highly conserved, at both DNA and protein levels, displaying many of the hallmarks of Hsps and sharing 80–96% of overall amino acid identities with homologous sequences from other diptera. The changes are mainly concentrated in the C-terminal domain of the protein. Phylogenetic analysis was consistent with the known classification of insects. The Hsp70 gene was located by in situ hybridization in region III-3A at the third polytene chromosome, a locus activated upon heat shock as shown by RNA pol II binding. As *C. riparius* is widely used in aquatic ecotoxicology testing, we studied Hsp70 gene induction in fourth instar aquatic larvae submitted to heat shock and selected environmental pollutants classified as potential endocrine disruptors. RT-PCR analysis showed that Hsp70 mRNA levels increased significantly ($p<0.05$) after short-term acute exposures to a temperature shift (HS), cadmium chloride (Cd), butyl benzyl phthalate (BBP), diethylhexyl phthalate (DEHP), bisphenol A (BPA), 4-nonylphenol (NP) and ethinylestradiol (EE). However, neither pentachlorophenol (PCP) nor tributyltin (TBTO) treatments were able to activate the Hsp70 gene. The cognate form, Hsc70, was also analysed and, unlike Hsp70, was not altered by any of the different treatments assayed. Moreover, at the times tested, there was no significant mortality of the larvae. The rapid upregulation of the Hsp70 gene suggests that it is sensitive and selective for different environmental pollutants, and could be used as an early molecular endpoint in ecotoxicological studies.

1. Introduction

Several thousand anthropogenic chemicals are continuously released into the natural environment, and all organisms are challenged by events that cause acute or chronic stress. Gene–environment interactions play a critical role in these processes. Environmental toxicants can trigger biological effects at the organism level only after initiating biochemical and cellular events. The cellular response to stress is characterized by the activation of genes involved in cell survival to counteract the physiological disturbance induced by physical or chemical agents. Cells activate a set of genes, called heat-shock genes as they were discovered by temperature insults, which mediate protective responses to temperature, radiation and environmental contaminants (Morimoto, 1998). It is widely accepted that the family of heat-shock proteins counteracts cellular stress and its associated damage (Feder and Hoffman, 1999; Nolen and Morimoto, 2002). Hsps are suitable as an early warning bioindicator of environmental hazard, because of their sensitivity to even minor changes in cellular homeostasis and their conservation along the evolutionary scale. Currently, their potential use for predicting the toxicity of chemicals is being actively investigated (De Pomerai, 1996; Gupta et al., 2010). Attention is also now being focused on modulating the expression of this group of proteins for the treatment of a wide variety of human diseases (Powers and Workman, 2007).

Among Hsps, the Hsp70 family represents one of the most highly conserved proteins identified to date, and has constitutive as well as regulated members in all the organisms examined (Mayer and Bukau, 2005). Hsp70 is one of the most abundantly induced proteins under a variety of stress conditions, while Hsc70 members are constitutively expressed under normal growth conditions. Most experimental work on the Hsp70 family has aimed to clarify the molecular mechanism of the chaperon system, with considerable recent progress in understanding the family's diverse functions in cells related to signalling pathways and protein homeostasis (for a review see Young, 2010). In addition, it is worth noting the recent advances in the molecular description of Hsp70 genes in a variety of species, as well as their evaluation in response to environmental stressors and toxicants (Dang et al., 2010; Karouna-Renier and Rao, 2009; Rhee et al., 2009; Ming et al., 2010; Simoncelli et al., 2010; Sinha et al., 2010; Su et al.,

Abbreviations: aa, amino acids; BBP, butyl benzyl phthalate; bp, base pairs; BPA, bisphenol A; DEHP, diethylhexyl phthalate; EE, ethinylestradiol; NP, 4-nonylphenol; PCP, pentachlorophenol; RACE, rapid amplification of cDNA ends; TBTO, tributyltin oxide.

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2010; Waagner et al., 2010; Zhanng and Denlinger, 2010). It is now clear that compounds, including pesticides, metals and a variety of organic chemicals, are able to induce the production of some Hsps. However, depending upon the type of inducer there are variations in the pattern, magnitude, kinetics and duration of Hsp expression, which are still not clearly understood. Our knowledge of the underlying mechanisms governing the activation of Hsp70 genes is still far from complete. This environmental perspective adds further interest for studying the relevance of the Hsp70 gene as a toxicological endpoint after exposure to different environmental pollutants (Gupta et al., 2010).

Invertebrates, especially arthropods such as insects and crustaceans, constitute the vast majority of animal species on earth. Yet, they have received much less attention than vertebrates regarding the potential toxicity of most man-made chemicals. It is well known that aquatic insects are sensitive bioreporters of xenobiotic contamination, as exposure occurs during critical developmental stages, such as embryogenesis, larval development and pupation. The midge *Chironomus riparius* is an EPA- and OECD-approved test organism widely used in environmental toxicology (EPA, 1996; OCDE 2001). Chironomid larvae are employed in aquatic toxicity studies because of their ecological relevance in freshwater ecosystems and their association with benthic sediments, where the accumulation of many pollutants takes place. Survival tests and changes in developmental parameters are used in most studies to evaluate toxicity responses. Larval mouthpart deformities also function as indicators of anthropogenic stress (Martinez et al., 2003). Moreover, the giant polytene chromosomes from the salivary gland cells are a particularly suitable material for analysing the genotoxic effects of these compounds (Michailova et al., 2006). Although such studies have provided valuable data, novel molecular endpoints should also be used, in combination with classical reproductive endpoint and life-cycle testing, to increase our understanding of the mechanisms and modes of action of xenobiotics. The genes coding Hsps have recently acquired great relevance, and Hsp70 has been sequenced and evaluated as a biomarker of exposure to metals and insecticides in some species of chironomids, such as *Chironomus yoshimatsui* and *Chironomus dilutus* (Yoshimi et al., 2002; Karouna-Renier and Rao, 2009). The aim of the present study was to characterize the Hsp70 gene in *C. riparius* and to investigate transcriptional regulation of this gene under control and different stressful conditions, including temperature shifts and exposures to metals, insecticides and different organic chemicals classified as potential endocrine disrupting compounds (EU-Strategy for Endocrine Disruptors/Environment-Endocrine Disruptors Website). Developmental and reproductive impairments have been clearly demonstrated for these chemicals in a number of species, but relatively little is known about the subtle effects at the molecular level. Moreover, gene expression profiles could be a powerful new endpoint for ecotoxicological studies (Snell et al., 2003).

2. Material and methods

2.1. Animals and treatments

The experimental animals were fourth instar larvae from the midge *C. riparius*. They were obtained from laboratory cultures; larvae were originally collected from natural populations in a non-polluted area of Valencia (Spain), and reared under standard laboratory conditions according to toxicity testing guidelines (US-EPA, 1996; OECD, 2001). Larvae were grown from egg masses in an aqueous culture medium (0.5 mM $CaCl_2$, 1 mM NaCl, 1 mM $MgSO_4$, 0.1 mM NaHCO3, 0.025 mM KH_2PO_4, and 0.01 mM $FeCl_3$) supplemented with nettle leaves, commercial fish food, and cellulose tissue in polyethylene tanks (500 mL). Cultures were maintained under constant aeration at 20 °C and under standard light–dark periods (16L:8D). For experimental treatments, the larvae were exposed to the chemicals diluted in a culture medium for 24 h with constant aeration at 20 °C in glass recipients (200 mL). No food or substrate was provided during exposure. Dose selection was based on results from previous studies in *Chironomus* sp. and other arthropods. Fourth instar larvae were submitted to 10 mM cadmium chloride (Cd) (Fluka), 1 mg/L butyl benzyl phthalate (BBP) (Aldrich), 0.01 μg/L diethylhexyl phthalate (DEHP), 1 μM pentachlorophenol (PCP) (Aldrich), 3 mg/L bisphenol A (BPA) (Aldrich), 10 mg/L 4-nonylphenol (NP) (Fluka), 1 ng/L tributyltin oxide (TBTO) (Aldrich), and 5 mg/L ethinylestradiol (EE) (Sigma), nominal concentrations. For temperature treatments, larvae were heat-shocked at 35 °C for 120 min in a preheated and aerated cultured medium, as described previously (Morcillo et al., 1988). Each treatment consisted of at least three replicates, and three independent experiments were performed in each analysis using samples from three different control egg masses. The control larvae used in each case were exposed to the same concentration of solvent as the corresponding treatment and were also measured in triplicate. Larvae were stored at −80 °C until RNA isolation was carried out.

2.2. RNA isolation

Total RNA was extracted from control and exposed fourth instar larvae (ten animals for each experiment) using a guanidine isothiocyanate based method, performed with a commercial kit (Trizol, Invitrogen) according to the manufacturer's protocol. Briefly, frozen larvae were homogenated in one volume of Trizol and left for 5 min at room temperature. Then, 0.2 volumes of chloroform were added to each sample, mixed and left for 5 min at room temperature. Subsequently, the samples were centrifuged for 15 min at 4 °C and 15,000 *g*. Following transfer of the aqueous phase, the RNA was finally recovered by isopropyl alcohol precipitation (0.5 v/v), washed with 70% ethanol, and resuspended in DEPC water. The quality and quantity of total RNA were determined by agarose electrophoresis and absorbance spectrophotometry (Biophotomer Eppendorf), and purified RNA finally stored at −20 °C.

2.3. Amplification of HSP70

Total RNA extracted from *C. riparius* larvae, exposed for 2 h at 35 °C heat shock, was used for Hsp70 amplification. Based on the conserved sequences of Hsp70 genes from closely related species, two pairs of primers, Hsp70 (201-220) and Hsp70 (1006-987) (Table 1), were designed to amplify an Hsp70 cDNA fragment (806 bp) from *C. riparius*, while Hsp70 (852-873) and Hsp70 (2094-2076) were designed to amplify another Hsp70 cDNA fragment (1243 bp) from *C. riparius*. Cycling parameters for PCR amplification were one cycle of 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 1.5 min, with a final extension step at 72 °C for 10 min. The PCR products were purified (ExoSAP-kit One Step PCR Clean-up no. US78200, GE Healthcare) and sequenced with the primers [Hsp70 (201-220), Hsp70 (242-226), Hsp70 (341-319), Hsp70 (852-873), Hsp70 (1006-987), Hsp70 (1974-2001) and Hsp70 (2094-2076)] detailed in Table 1.

2.4. 5′ and 3′ RACE

The full-length sequence of Hsp70 was determined using 5′ and 3′ RACE (Rapid Amplification of cDNA Ends), using commercial kits (Invitrogen) and following the procedures described by the manufacturer. New gene-specific primers were designed, based on sequence information obtained from the internal fragments. The sequences of all gene-specific primers used for RACE are given in Table 1.

For the 3′ end RACE PCR, a cDNA template was obtained as described above, and a PCR was performed with gene-specific primer Hsp703′ (1) and an adapter primer AUAP (RACE kit, Invitrogen). The PCR conditions were one cycle of 94 °C for 5 min, followed by 35 cycles