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The endocrine disruptor bisphenol A increases the expression of HSP70 and ecdysone receptor genes in the aquatic larvae of *Chironomus riparius*

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Abstract

Bisphenol A (BPA) is an endocrine disruptor that can mimic the action of estrogens by interacting with hormone receptors and is, therefore, potentially able to influence reproductive functions in vertebrates. Although information about the interaction with the endocrine systems in invertebrates is limited, it has also been shown its effect on reproductive and developmental parameters in these organisms. As little is known about its mechanism of action in aquatic invertebrates, we have examined the effects of BPA on the expression of some selected genes, including housekeeping, stress-induced and hormone-related genes in *Chironomus riparius* larvae, a widely used organism in aquatic ecotoxicology. The levels of different gene transcripts were measured by Northern blot or by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). Exposure to BPA (3 mg l^{-1}, 12–24 h) did not affect the levels of rRNA or those of mRNAs for both L11 or L13 ribosomal proteins, selected as examples of housekeeping genes involved in ribosome biogenesis. Nevertheless, BPA treatment induced the expression of the HSP70 gene. Interestingly, it was found that BPA significantly increases the mRNA level of the ecdysone receptor (EcR). These results show for the first time that exposure to endocrine disrupting chemicals, such as BPA, can selectively affect the expression of the ecdysone receptor gene suggesting a direct interaction with the insect endocrine system. Furthermore, this finding suggests a common way of BPA action, shared by vertebrates and invertebrates, through interaction with steroid hormone receptors. Our study adds a new element, the EcR, which may be a useful tool for the screening of environmental xenoestrogens in insects.

Keywords: Bisphenol A; Ecdysone receptor; HSP70; rRNA; Ribosomal proteins; *Chironomus*

1. Introduction

In recent years, the potential adverse effects of endocrine disrupting chemicals (EDCs) in human health and ecosystems have become a major research issue. Bisphenol A (BPA) is one of the industrial compounds that have generated concerns due to its high production and widespread use in many consumer products. BPA is extensively used as a primary monomer in epoxy resins and polycarbonate plastics, as well as an antioxidant in plasticizers and as an additive in other plastics (Cousins et al., 2002). Accumulating evidence indicates that BPA is a xenoestrogen that, potentially, can have adverse effects on humans as well as wildlife (Crain et al., 2007, for a review). Bisphenol A acts as a selective estrogen receptor modulator in mammals (Welshons et al., 2006, for a review). A wide range of significant effects has been reported on reproduction and development, the immune system and the nervous system in experimental animals, mainly rodents (ÓConnor and Chapin, 2003). In other organisms studied, vertebrates

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and invertebrates, BPA causes estrogen-like developmental effects. Some of the effects reported are: a reversal in gonadal sex and alteration in gonadal histoarchitecture in *Caiman latirostris* (Stoker et al., 2003); feminization in *Xenopus* tadpoles (Levy et al., 2004); and superfeminization in the snail *Marisa cornuarietis* (Oehlmann et al., 2006).

The total amount of BPA released into the environment has been estimated, and it has been assumed that over 90% is released into water. Therefore, its predicted concentration in water and sediments has become a primary environmental concern (Cousins et al., 2002). Despite the importance of BPA toxicity in aquatic ecosystems, its effect in aquatic organisms has been less studied in comparative terms (Kang et al., 2007, for a review). In fishes, reproductive effects, such as the inhibition of gonadal growth and egg production, have been described in fathead minnows after long-term exposure to BPA (Sohoni et al., 2001). In addition, a significant increase in estrogen metabolism has been found in the kidney of lake trout (Jurgella et al., 2006). Only a few studies have been devoted to analyse the effects of BPA in aquatic invertebrates (Segner et al., 2003).

The midge *Chironomus* is widely used in aquatic toxicology and is considered to be an appropriate test species for research about potential endocrine disrupting substances (Taenzler et al., 2007). It is particularly suitable for studying the effects of BPA, as it has been estimated that its accumulation and long half-life occur in sediment, which is the habitat of the midge larvae. Moreover, the most comprehensively described endocrine system within invertebrates is that of insects. Previous studies in *Chironomus riparius* have shown that BPA exposure delayed moulting and increased mouthpart deformities (Watts et al., 2001, 2003). It was selected as a reference organism in the EU-funded IDEA project to evaluate the endocrine disrupting effects in aquatic invertebrates; among other compounds analysed, BPA delayed emergence times, suggesting some disruption of normal development in *Chironomus* (Segner et al., 2003). BPA also affected yolk protein content, suggesting some alterations in vitellogenesis (Hahn et al., 2002). More recently, the effect of BPA has also been described in HSP and haemoglobin genes, as well as in some enzyme activities, such as catalase and glutathione-S-transferase (Lee et al., 2006; Lee and Choi, 2007).

The present study was undertaken to determine the early molecular and subcellular effects of BPA in *C. riparius* larvae. The effects of BPA exposure were examined in the expression of different selected genes at the transcriptional level. The analysis included housekeeping genes, such as rDNA, L11 and L13 ribosomal protein genes, stress-induced genes, such as HSP70, and hormone-related genes, such as the ecdysone receptor gene (EcR). Our study provides evidence for the changes in the pattern of gene expression induced by BPA exposure. The finding of the upregulation of the ecdysone receptor (EcR) gene may suggest for the first time a mechanism for the endocrine mediating effects of BPA in insects.

2. Materials and methods

2.1. Animals and treatments

The experimental animals were fourth instar larvae from the midge *C. riparius*. They were originally collected from natural populations in Valencia (Spain), and reared under standard laboratory conditions. Larvae were grown in culture medium (0.5 mM $CaCl_2$, 1 mM NaCl, 1 mM $MgSO_4$, 0.1 mM $NaHCO_3$, 0.025 mM KH_2PO_4, 0.01 mM $FeCl_3$) supplemented with nettle leaves, commercial fish food, and cellulose tissue. Cultures were maintained under constant aeration at 18 °C and under standard light-dark periods. For BPA treatments, the larvae were exposed to 3 mg l^{-1}, 6 mg l^{-1}, 9 mg l^{-1}, 12 mg l^{-1} BPA diluted in culture medium for 12 and 24 h with constant aeration at 18 °C. Dose selection was based on results from previous studies in *Chironomus* (Hahn et al., 2002; Segner et al., 2003; Watts et al., 2003). No food or substrate was provided during exposure. Each treatment consisted of at least three replicates, and three independent experiments were performed in each analysis.

2.2. RNA isolation

Total RNA was extracted from control and BPA-exposed fourth instar larvae (10 animals for each experiment) using a guanidine isothiocyanate based method, performed with a commercial kit (TRIzol, Invitrogen) according to the manufacturer's protocol. Finally, the RNA was recovered by isopropyl alcohol precipitation and resuspended in DEPC water.

2.3. RT-PCR

Reverse transcription was performed with 1 μg of total RNA. An oligo dT primer (Invitrogen) was used with Superscript II enzyme (Invitrogen), following the manufacturer's instructions. The cDNAs obtained were used as templates for PCR reactions with gene-specific primers for actin, and the previously identified L11, L13 and HSP70 genes (Martínez-Guitarte et al., 2007). In the case of EcR, a search in the FASTA invertebrate database was carried out to detect conserved regions. A partial clone was obtained by using PCR primers designed from the conserved insect sequences in the EcR receptor gene from *Chironomus tentans* (accession number S60739). The resulting PCR products were subsequently sequenced and a 240 bp fragment was obtained that had 97% base sequence identity with *C. tentans* EcR. The sequences of the oligonucleotide primers were: actin forward 5′-GATGAAGATCCTCACCGAACG-3′; actin reverse 5′-CCTTACGGATATCAACGTCGC-3′; RPL11 forward 5′-AGATCCCGTAAGCTTTGCC-3′; RPL11 reverse 5′-CATACTTCTGTTGGAACC-3′; RPL13 forward 5′-AAGCTGCTTTCCCAAGAC-3′; RPL13 reverse 5′-TTGGCATAATTGGTCCAG-3′; HSP70 forward 5′-CATGTGAACGAGCCAA-