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Transcriptional changes induced by *in vivo* exposure to pentachlorophenol (PCP) in *Chironomus riparius* (Diptera) aquatic larvae

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ABSTRACT

Pentachlorophenol (PCP) has been extensively used worldwide as a pesticide and biocide and is frequently detected in the aquatic environment. In the present work, the toxicity of PCP was investigated in *Chironomus riparius* aquatic larvae. The effects following short- and long-term exposures were evaluated at the molecular level by analyzing changes in the transcriptional profile of different endocrine genes, as well as in genes involved in the stress response and detoxification. Interestingly, although no differences were found after 12- and 24-h treatments, at 96-h exposures PCP was able to induce significant increases in transcripts from the *ecdysone receptor gene (EcR)*, the *early ecdysone-inducible E74 gene*, the *estrogen-related receptor gene (ERR)*, the *Hsp70 gene* and the *CYP4G gene*. In contrast, the *Hsp27 gene* appeared to be downregulated, while the *ultraspiracle* gene (*usp*) (insect ortholog of the retinoid X receptor) was not altered in any of the conditions assayed. Moreover, Glutathione-S-Transferase (GST) activity was not affected. The results obtained show the ability of PCP to modulate transcription of different biomarker genes from important cellular metabolic activities, which could be useful in genomic approaches to monitoring. In particular, the significant upregulation of hormonal genes represents the first evidence at the genomic level of the potential endocrine disruptive effects of PCP on aquatic invertebrates.

1. Introduction

Pentachlorophenol (PCP) is an organochlorine compound used worldwide, mainly as a pesticide and wood preservative. It is a widespread, ubiquitous environmental contaminant (Geyer et al., 1987; WHO, 1987; Muir and Eduljee, 1999; Heudorf et al., 2000; Ge et al., 2007; Zheng et al., 2011, 2012; Montaño et al., 2013). The use of this xenobiotic is restricted in Europe and the USA, and the United States Environmental Protection Agency (US-EPA) has listed it as a priority pollutant due to its slow and incomplete biodegradation (Gupta et al., 2002; Hanna et al., 2004). This Persistent Organic Pollutant (POP) is stable in the aquatic environment and sediments have shown the highest PCP concentrations (Zheng et al., 2012; Li et al., 2013), which have been related to its long half-life with almost no degradation in sediments (WHO, 1987). PCP is highly toxic for most organisms; the primary mechanisms of toxic action proposed are the uncoupling of substrate oxidation from ATP in mitochondria and the provoking of oxidative stress in cells and tissues (Xu et al., 2014a). In humans, this compound affects the immune and endocrine systems, kidneys, lungs and liver; neurotoxicity and carcinogenicity have also been suggested (Proudfoot, 2003; Hurd et al., 2012). Various studies have also reported that it induces the following: production of Reactive Oxygen Species (ROS); lipid peroxidation (Dong et al., 2009); cell morphological alterations (Chen et al., 2004a; Yang et al., 2005); mitochondrial dysfunction and lysosomal membrane damage (Fernández Freire et al., 2005); reproduction (Zha et al., 2006); and cell death (Wang et al., 2001; Wispriyono et al., 2002; Chen et al., 2004b; Yang et al., 2005; Dong et al., 2009). Furthermore, PCP inhibited oocyte maturation in zebrafish (Tokumoto et al., 2005) and decreased the production of steroid hormones and the downregulation of steroidogenic gene expression in human cell culture (Ma et al., 2011). In arthropods, PCP teratogenesis has been demonstrated by observing the deformation of the mentum in *Chironomus plumosus*

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larvae (Song, 2007). More recently, a significant increase in DNA damage has also been observed when *C. riparius* were exposed to PCP (Martínez-Paz et al., 2013).

The potential adverse effects of Endocrine Disrupting Chemicals (EDCs), those xenobiotics able to interact with hormones altering the endocrine regulation of essential physiological functions, have become a major research interest. The impact of PCP on the endocrine system has received much attention in recent years. Although there is still limited information, it has been found that PCP might disrupt the thyroid endocrine system, and using *in vitro* and *in vivo* assays in *Xenopus laevis*, it has been shown to have T3-antagonist activity (Sugiyama et al., 2005). Similar results have been obtained using the *in vitro* assay with rat pituitary GH3 cells and *in vivo* assays with zebrafish embryos (Guo and Zhou, 2013). Furthermore, PCP alters plasma thyroid hormone levels, as well as the expression of genes associated with thyroid hormone signaling and metabolism in the hypothalamic–pituitary–thyroid (HPT) axis and liver in zebrafish (Yu et al., 2014). Recent studies have shown that the vitellogenin mRNA expression increased when female rare minnows (*Gobiocypris rarus*) were exposed to PCP (Zhang et al., 2014). In humans, a negative association has been reported between maternal plasma PCP levels and cord plasma free T4 concentrations in neonates (Dallaire et al., 2009). The potential disruption caused by PCP to the thyroid endocrine system has raised great concern over its adverse environmental health risks. However, currently there is little information available about its interaction with endocrine systems in invertebrates, and little is known about its mechanism of action. To date, only a few studies have been devoted to analyzing the effects of PCP on aquatic invertebrates.

The aquatic larvae of the midge *Chironomus riparius* are widely used as a test organism for the assessment of aquatic toxicology (US-EPA, 2000; OECD, 2013). The study of the taxonomic composition of chironomid larvae and the percentage of deformities in mouthparts, mainly in the mentum, are used in biomonitoring programs to obtain information about the levels of organic and chemical pollution of aquatic ecosystems (Martinez et al., 2003). Moreover, they are being increasingly employed for toxicity testing using molecular endpoints. In recent years, some genes have been described as biomarkers for different aquatic contaminants, including among others, those for heat shock proteins (HSPs), ribosomal proteins, nuclear receptors and cytochrome P450 genes (Londoño et al., 2007; Martínez-Guitarte et al., 2007; Planelló et al., 2007, 2008, 2010, 2011; Park and Kwak, 2008, 2009, 2010; Park et al., 2009; Nair et al., 2011, 2013; Morales et al., 2011, 2013; Martínez-Paz et al., 2012, 2014). The aim of the present study was to investigate the toxicity and molecular effects of PCP in *C. riparius*, focusing on the expression of different endocrine-related genes, particularly receptor genes and transcription factors acting in the intracellular signaling of steroids in larvae. We analyzed the impact of PCP on the activity of the *EcR*, *usp* and *ERR* genes, codifying for three hormonal receptors. The estrogen-related receptor (ERR), the ecdysone receptor (EcR) and the ultraspiracle (USP; ortholog of the retinoid X receptor) of the invertebrate ecdysozoans belong to the nuclear receptor super family (Köhler et al., 2007), and the EcR and USP appear to be regulated by ecdysone pulses during insect development and metamorphosis (Rauch et al., 1998; Gilbert and Warren, 2005). Analysis was also undertaken for an early ecdysone-responsive gene (*E74*), coding for a transcription factor that in turn is regulated by the ecdysone receptor. In addition, the effects were also examined on genes related to the stress response, such as the *Hsp70* and *Hsp27* genes, and on some parameters related with the detoxification routes, such as the *CYP4G* gene and Glutathione-S-Transferase (GST) activity. This study offers the first evidence of the genomic effects of PCP on the endocrine pathway in aquatic invertebrates, which would be of interest for further selection of gene biomarkers for environmental risk assessment.

2. Material and methods

2.1. Animals and treatments

The experimental animals were fourth instar larvae from the midge *Chironomus riparius*. They were originally collected from natural populations in a non-polluted area of Valencia (Spain), and reared under standard laboratory conditions for several generations according to toxicity testing guidelines (US-EPA, 2000; OECD, 2013). 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 (TetraMin) and cellulose tissue. Cultures were maintained under constant aeration at 20 °C and under standard light-dark periods 16:8. For experimental treatments, larvae were exposed to the chemical diluted in culture medium for 12, 24 and 96 h with constant aeration at 20 °C. In 96-h exposures, culture medium was renewed every 24 h and supplemented with 3 mg of commercial fish food at 48 h. Concentrations were chosen based on former experiments under similar exposure scenarios in *Chironomus* or related species. Fourth instar larvae ($n = 30$ per replica) were submitted to 25, 250, 1000, 1500, 2000 and 2500 μg/L of pentachlorophenol (PCP) (Aldrich). Each treatment consisted of three independent experiments performed in each analysis using samples from three different egg masses. The control larvae used in each case were exposed to the same concentration of solvent (ethanol 0.001%) as the corresponding treatment and were also measured in triplicate. Larvae exposed to 25 and 250 μg/L (94 and 940 nM, respectively) of PCP and untreated larvae were stored at −80 °C until RNA and protein isolation was carried out.

2.2. Toxicity test

Observations on the survival of the larvae were made after 24, 48, 72 and 96 hours, with death of individuals as an endpoint. Larvae were considered dead when they did not move in response to being probed with forceps.

2.3. RNA isolation

Total RNA was extracted from control and exposed fourth instar larvae using a guanidine isothiocyanate-based method, performed with a commercial kit (TRIZOL, Invitrogen) according to the manufacturer's protocol. Subsequently, RNA was treated with RNase-free DNase (Roche), followed by phenolization. The quality and quantity of total RNA were determined by agarose electrophoresis and absorbance spectrophotometry (Biophotomer Eppendorf). Finally, purified RNA was stored at −80 °C.

2.4. Real time RT-PCR

Reverse transcription was performed with 0.5 μg of the isolated RNA and 0.5 μg oligonucleotide dT_{20} primer (Sigma) was used with 100 units of M-MLV enzyme (Invitrogen), in a final volume of 20 μL. A total of 25 ng of cDNA was obtained and used as a template for the Polymerase Chain Reaction (PCR). Quantitative real-time PCR (qRT-PCR) was used to evaluate the mRNA expression profile of the *EcR*, *usp*, *E74*, *ERR*, *Hsp70*, *Hsp27* and *CYP4G* genes in control and under PCP exposures. The qRT-PCR was performed with a CFX96 thermocycler (Bio-Rad) and SsoFast EvaGreen Supermix (Bio-Rad). The qRT-PCR was run in the following cycling conditions: initial denaturation at 95 °C for 3 min and 35 cycles of 95 °C denaturation for 5 s; 58 °C annealing for 15 s; and 65 °C elongation for 10 s. *C. riparius* does not have the relevant genetic sequence information in databases and the typically endogenous reference genes used in this species are *actin-β*, *Glyceraldehyde 3-phosphate*