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Transcriptional activity of detoxification genes is altered by ultraviolet filters in *Chironomus riparius*

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ABSTRACT

Ultraviolet (UV) filters are compounds used to prevent the damage produced by UV radiation in personal care products, plastics, etc. They have been associated with endocrine disruption, showing anti-estrogen activity in vertebrates and altering the ecdysone pathway in invertebrates. Although they have attracted the attention of multiple research teams there is a lack of data about how animals activate detoxification systems, especially in invertebrates. Here, analysis of the effects of two UV filters, benzophenone-3 (BP3) and 4-methylbenzylidene camphor (4MBC), on the transcriptional activity of nine genes covering the three steps of the detoxification process has been performed. Four cytochrome P450 genes belonging to different members of this family, five GST genes, and the multidrug resistance protein 1 (MRP1) gene were studied by RT-PCR to analyze their transcriptional activity in fourth instar larvae exposed to the UV filters for 8 and 24 h. The obtained results show a differential response with downregulation of the different Cyp450s tested by 4MBC while BP3 seems not to modify their expression. On the other hand, some of the GST genes were affected by one or other of the filters, showing a less homogenous response. Finally, *MRP1* was activated by both filters but at different times. These results demonstrate for first time that UV filters alter the expression of genes involved in the different steps of the detoxification process and that they can be processed by phase I enzymes other than Cyp450s. They also suggest that UV filters affect biotransformation processes, compromising the ability of the individual to respond to chemical stress, so further research is needed to know the extent of the damage that they can produce in the resistance of the cell to chemicals.

1. Introduction

Ultraviolet (UV) filters are chemicals commonly used in different industrial products including personal care products (PCPs), plastics, and varnish. They are regulated and must be declared in cosmetics but remain essentially unidentified in technical products (Regulation (EC) no. 1223/2009). In this sense, it is important to note that an analysis of occurrence of benzophenones in personal care products of China and US has shown that benzophenone-3 (BP3) can be found in 81% of the samples and reaching concentrations as high as 0.148% (Liao and Kannan, 2014). In the same study they estimated the daily intake of this UV filter in 0.978 and 24.4 μg/day for Chinese and American adult women. UV filters have been detected in aquatic environmental samples (Fent et al., 2010; Grabicova et al., 2013; Sánchez-Quiles and Tovar-Sánchez, 2015; Tarazona et al., 2010), tap water, and treated sewage sludge (Díaz-Cruz et al., 2012; Gago-Ferrero et al., 2011). They have been found from ng/L to μg/L in wastewater treatment effluent (reviewed in Montes-Grajales et al., 2017, Kasprzyk-Hordern et al., 2009). In India, benzophenones can be found in a mean concentration of 351 and 163 ng/L in influent and effluent of wastewater treatment plants (WWTPs) (Karthikraj and Kannan, 2017). Benzophenone-3 and other benzophenones have been also detected in sediments of Chinese and American rivers and in sludge of Chinese WWTPs in ng/g ranges (Zhang et al., 2011). Recently, it has been calculated that almost 11% of the BP3 production in the US reach WWTPs while 3% of it were emitted through WWTP discharges (Wang and Kannan, 2017). On the other hand, it has been estimated that the input of four different UV filters can be as high as 118 g of 2-ethyl-hexyl-4-trimethoxycinnamate (EHMC), 49 g of 4-methyl-benzilidine-camphor (4MBC), 69 g of BP3, and 28 g of octocrylene (OC) per 10,000 people per day (Balmer et al., 2005). Animals from aquatic ecosystems incorporate them as they are found in fishes and aquatic macroinvertebrates (Buser et al., 2006; Calafat et al., 2008). Endocrine disruptor activity has been demonstrated in vertebrates (Díaz-Cruz and Barceló, 2009; Schlumpf et al., 2001) and invertebrates (Ozáez et al., 2014, 2016a, 2016b). Considered as emergent contaminants, their levels are increasing in the environment but studies about effects other than endocrine disruption are still scarce in vertebrates and invertebrates. Stress response has been

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demonstrated to be altered by these compounds (Gao et al., 2013; Ozáez et al., 2016b, 2016c; Rodríguez-Fuentes et al., 2015) but detoxification, a common event in response to most toxicants, has not been studied. Detoxification mechanisms usually imply three phases. Phase I involves enzymes such as cytochrome P450 oxidases introducing reactive or polar groups into the compound to decrease toxicity (Schuetz, 2001). These modified compounds are then conjugated to polar compounds such as glutathione or glucuronic acid in phase II reactions, increasing solubility to ease their elimination. Transferase enzymes such as glutathione S-transferases are responsible for these modifications (Jancova et al., 2010). Finally, phase III involves efflux transporters like ABC transporters (Dermauw and Van Leeuwen, 2014) that pump the modified compounds out of cells.

Chironomus riparius Meigen 1804 (syn. *Chironomus thummi* Kieffer 1911) is an organism frequently used in aquatic ecotoxicology studies because of its aquatic larvae and association with benthic sediments where most pollutants accumulate. Furthermore, it is an ecologically relevant organism in freshwater environments as an important food source for fish and other animals (Armitage et al., 1995). Several genes related to the detoxification system have been described in *C. riparius*. Two different cytochrome P450 genes belonging to families 4 and 9 (Martínez-Paz et al., 2012; Nair et al., 2013a) and several glutathione S-transferases (Nair and Chung, 2015; Nair et al., 2011, 2013b) have been previously identified. It has been demonstrated that a number of toxicants, like cadmium, silver nanoparticles, zinc nanoparticles, nonylphenol, and bisphenol A, can alter the expression of these genes (Martínez-Paz et al., 2012; Nair and Chung, 2015; Nair et al., 2011, 2013a). However, no studies involving analysis of the response of these genes to UV filters have been published so far. On the other hand, there has not been described in this species any member of the ABC transporter family involved in detoxification phase III.

UV filters are now extended in the industry and start to be an important environmental concern; however, we have poor knowledge about activation of detoxification mechanisms by the cell. Only a recent study has analyzed the biotransformation capability of different cell lines from humans and zebrafish (Le Fol et al., 2015). For invertebrates no study centered on this matter has been published. Given this limited ecotoxicological information, the purpose of this study was to analyze the activation of genes related to detoxification mechanisms by two different UV filters, benzophenone-3 and 4-methylbenzylidene camphor, in *C. riparius*. BP3, also known as oxybenzone, is a common component of sunscreen lotions and plastic belonging to the class of aromatic ketones. 4MBC is an organic camphor derivative used in PCPs as a protector against UV B radiation. Four Cyp450s belonging to different families (4, 6, 9, and 12), five GSTs from four different classes (delta, epsilon, omega, and theta), and the multidrug resistance protein 1 (MRP1) genes have been analyzed in the acute response of fourth instar larvae to elucidate the involvement of these genes in detoxification of two UV filters, BP3 and 4MBC.

2. Material and methods

2.1. Chemicals

The UV filters 3-(4-methylbenzylidene) camphor (CAS no. 36861-47-9, purity ≥ 98%) and benzophenone-3 (2-hydroxy-4-methoxybenzophenone; CAS no. 131-57-7, purity ≥ 98%) were purchased from Sigma-Aldrich (Spain). Stock solutions were made in absolute ethanol and stored in the dark at 4 °C.

2.2. Animals

The experimental animals were aquatic larvae of the midge *C. riparius*. Stock cultures are maintained in the laboratory of Biology and Environmental Toxicology (UNED) from natural populations of midge larvae originally collected in a non-polluted area of Valencia (Spain) and reared under standard laboratory conditions according to OECD guidelines (OECD, 2001). Larvae were grown from egg masses in aqueous 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) supplemented with nettle leaves, commercial fish food (TetraMint, Germany), and cellulose tissue in polyethylene tanks. Cultures were maintained under constant aeration at 20 °C and under standard light–dark periods (16 L: 8D).

2.3. Treatment

Fourth instar larvae were exposed to each chemical diluted in 50 mL culture medium for 8 and 24 h in glass vessels (250 mL). Nominal concentrations of 0.1 and 1 mg/L of the UV filters were chosen following previous published data (Ozáez et al., 2016a, 2016b). UV filters were added directly to the aqueous culture medium from the stock solutions. To prevent losses by adsorption during treatments, no sediment, cellulose tissue, or food were added. The treatments were carried out in the absence of light, due to their photodegradation. Non-treated control larvae were exposed to the same concentration of solvent (0.02% ethanol). Three independent experiments were performed for each condition using samples from different egg masses. Three larvae from each experiment were frozen and stored at −80 °C for RNA extraction so the final number of larvae analyzed for each condition was n = 9.

2.4. Sequence identification

A file including all the sequences from project SRX147945 (Marinković et al., 2012) was downloaded from the Sequence Read Archive database and used to run a systematic search in the GenBank database with the Blast2GO tool (Conesa et al., 2005). The conditions of the search were tblastx program, non-redundant database, and Blast ExpectValue of 1.0E-3. Those sequences identified as being from the genes of interest were employed to perform a second search in Blast using the blastn program and Transcriptome Shotgun Assembly database. The sequences identified were translated with SnapGene software (GSL Biotech LLC) and the proteins analyzed to confirm that they corresponded to the gene selected.

2.5. RNA isolation

Total RNA was extracted from each control and experimental individual fourth instar larva using a guanidine isothiocyanate-based method performed with TRIzol, a commercial kit (Invitrogen, Germany), according to the manufacturer's protocol. Afterwards, RNA was treated with RNase-free DNase (Roche, Germany) and, subsequently, a phenol: chloroform: isoamyl alcohol (Fluka, Spain) extraction using Phase Lock Light tubes (5prime, Spain) was carried out. The quality and quantity of total RNA were determined by agarose electrophoresis and absorbance spectrophotometry (BioPhotometer, Eppendorf, Germany). Finally, purified RNA was stored at −80 °C.

2.6. cDNA synthesis and real-time RT-PCR

Aliquots of 1 μg of total RNA were reverse-transcribed using 100 units of M-MLV enzyme (Invitrogen, Germany) in the presence of 0.5 μg oligonucleotide dT20 primer (Sigma, Spain) and 0.5 mM dNTPs (Biotools, Spain) at 37 °C for 50 min in a final volume of 20 μL. This cDNA was used as the template for the polymerase chain reaction (PCR). Real-time PCR was used to evaluate the mRNA expression profile of genes in controls and treatments. The RT-PCR was performed with a CFX96 thermocycler (Bio-Rad, USA) using 0.5 units of DNA polymerase (Biotools, Spain), 0.4 mM dNTPs (Biotools, Spain), and 0.5X EvaGreen (Biotium, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), ribosomal protein L13 (rpL13), ribosomal protein S6 (rpS6), and phosphofructokinase (PhFK) genes were employed as