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The UV filter benzophenone 3 (BP-3) activates hormonal genes mimicking the action of ecdysone and alters embryo development in the insect *Chironomus riparius* (Diptera)

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ABSTRACT

Numerous studies have evaluated the endocrine effects of UV filters in vertebrates, but little attention has been paid to their possible hormonal activity in invertebrates. We examined the effects of benzophenone-3 (BP-3), one of the most common sunscreen agents, in *Chironomus riparius* (Insecta), a reference organism in aquatic toxicology. Salivary glands from larvae were treated with either the hormone ecdysone or BP-3 to compare the response of endocrine genes. It was found that BP-3 elicits the same effects as the natural hormone activating the expression of a set of ecdysone responsive genes. BP-3 also activated the stress gene *hsp70*. Interestingly, similar effects have been confirmed *in vivo* in embryos. Moreover, BP-3 also altered embryogenesis delaying hatching. This is the first demonstration of hormonal activity of UV filters in invertebrates, showing a mode of action similar to ecdysteroid hormones. This finding highlights the potential endocrine disruptive effects of these emergent pollutants.

1. Introduction

The presence of organic UV-absorbing chemicals in cosmetics and in numerous materials such as textiles and plastics has become common. It is estimated that over 10,000 tons of UV filters are produced annually for the global marked (Danovaro et al., 2008). The variety of such compounds, as well as the percentage of different filters added to industrial products, is increasing with the higher demand to prevent UV radiation damage. Due to their extensive use, UV filters are becoming ubiquitous environmental contaminants of increasing concern, given their bioaccumulation potential and the suspicion that they may act as endocrine disruptors. As UV filters are photostable and often lipophilic, they are relatively stable in the aquatic environment and, therefore, it is not surprising that residues of UV filters have been detected in tap water, wastewaters and treated sewage sludge (Gago-Ferrero et al., 2011), as well as in continental waters (Fent et al., 2010a) and coastal waters (Tovar-Sáchez et al., 2013). Organic UV filters will potentially bioaccumulate, and recent studies have reported the presence of these compounds in aquatic biota, such as fishes, aquatic birds and aquatic macroinvertebrates, with concentrations up to 2400 ng/g in fish from rivers and 701 ng/g in cormorants (Buser et al., 2006; Fent et al., 2010b). Benzophenones constitute one of the most common sunscreen agents in cosmetics. Because studies are scarce and limited to a few locations, we still know little about the extent and magnitude of their presence in aquatic environmental samples, but appreciable amounts of benzophenones have been detected in most samples with concentrations that varied largely. For BP-3, concentrations of 620 ng/L up to more than 3.3 μg/L that has been recently reported (Tarazona et al., 2010; Grabicova et al., 2013), and even higher levels 5.61 μg/L were found in raw drinking water in California, USA (Loraine and Pettigrove, 2006). Moreover, the presence of Benzophenone-3 (BP-3) was detected in 98% of urine samples in a survey of the US population (Calafat et al., 2008) and, in a more recent study, it was one of the most frequently found (83%) UV filters in the blood of adults (Zhang et al., 2013).

There is increasing evidence of the possible interaction of several UV filters with the endocrine system. The endocrine disrupting effects of some UV filters were initially described *in vitro* for human cells, as well as *in vitro* and *in vivo* tests in rodents (Schlumpf et al., 2001). Most UV filters subsequently investigated have been suspected of eliciting hormonal activity in vertebrates, in a similar range to that caused by other well-known endocrine disrupting

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compounds (EDCs) (Díaz-Cruz and Barceló, 2009, for a review). A large number of ultraviolet-absorbing compounds show estrogenic activities, both *in vivo* and *in vitro* test systems (Holbech et al., 2002; Schreurs et al., 2002; Mueller et al., 2003; Schlumpf et al., 2004; Klammer et al., 2005; Kunz et al., 2006; Kunz and Fent, 2006). Recent studies have demonstrated that not only estrogens but also different hormonal targets are affected by UV filters in mammals and fishes. Certain UV filters show antiandrogenic activities (Ma et al., 2003; Suzuki et al., 2005; Schreurs et al., 2005), while others are suspected of having thyroid-disrupting properties (Schmutzler et al., 2007; Axelstad et al., 2011).

To date, most attention has focused on the interaction of UV filters with sex-steroid hormones in mammals, because research about the adverse effects of UV filters has mainly concentrated on assessing the potential risk for humans. Although significantly less attention has been paid to the effects in invertebrates, there is also some evidence of the toxic effects and developmental or reproductive impairments of UV filters in these organisms (Fent et al., 2010b; Sieratowicz et al., 2011; Schmitt et al., 2008; Kaiser et al., 2012; Tovar-Sáchez et al., 2013). Nevertheless, the possible hormonal activity of these compounds on invertebrate endocrine systems still needs to be investigated. We recently published the first evidence suggesting that UV filters directly interfere with insect endocrine pathways, because exposure to 4-methylbenzylidene camphor (4-MBC), octyl-p-methoxycinnamate (OMC) and octyldimethyl-p-aminobenzoate (OD-PABA) induced significant overexpression of the ecdysone receptor gene in *Chironomus riparius* midge larvae (Ozáez et al., 2013). The ecdysone receptor is the cell mediator of ecdysone hormone action. This steroid hormone, together with the juvenile hormone, regulates development and metamorphosis in insects. Well-known endocrine disruptors, such as bisphenol A, phthalates, tributyltin and cadmium, have also shown the ability to activate the ecdysone receptor gene in this organism (Planelló et al., 2008, 2010, 2011; Nair and Choi, 2012; Morales et al., 2013). Therefore, these preliminary results suggest a common mode of action shared by UV filters and other well-known hormonal active substances, and indicate that the ecdysone pathway is a target of UV filters. Hence, in this work we aimed to undertake an in-depth analysis in *Chironomus riparius* of the effects of UV filters on the signaling pathway of the insect steroid hormone ecdysone.

Ecdysteroid hormones exert their function at the genomic level and regulate growth, development and reproduction in arthropods (Smagghe, 2009). Among invertebrates, the endocrine system of insects is one of the best studied and, in particular, the ecdysone genomic response has been thoroughly characterized in *Drosophila* and *Chironomus*; indeed, it was in this latter organism that the action of steroids hormones on DNA was first discovered (Hill et al., 2013). *Chironomidae* are insects with aquatic life stages (embryo, larva and pupa) and *Chironomus riparius* larvae are widely used in environmental research for testing pollutant toxicity in sediments and freshwater environments (EPA, 2000; OECD, 2004). They are among the most abundant macroinvertebrates in sediments and are ecologically relevant to aquatic food chains. This environmental perspective adds further interest for studying the potential effects of UV-absorbing chemicals in this organism. In this study, the effects of benzophenone-3 (BP-3), one of the most widely used sunscreen agents, and 20-hydroxy Ecdysone (20-Ec), the active form of the natural hormone, were compared focusing on the analysis of genes that act as transducers in the genomic response of the cells to the hormone. Ecdysone-like activity was demonstrated for BP-3, either in exposed glands isolated from larvae or in whole exposed embryos. Our results show that key regulatory genes activated by the hormone ecdysone are also sensitive to BP-3. This is the first direct evidence of the hormonal activity of UV filters in an invertebrate endocrine system.

2. Material and methods

2.1. Chemicals

The UV filter benzophenone-3 (BP-3, 2-hydroxy-4-methoxybenzophenone, CAS No. 131-57-7, purity ≥98%) and the hormone 20-Hydroxyecdysone (purity ≥ 93%) were purchased from Sigma–Aldrich (Germany). Stock solutions were made in ethanol and stored in the dark at 4 °C.

2.2. Animals

The test organism used was the midge *Chironomus riparius*. Stock cultures were originally collected from natural populations in a non-polluted area of Valencia (Spain), and maintained under standard laboratory conditions for several generations according to toxicity testing guidelines (EPA, 2000; OECD, 2004). Larvae were grown from egg masses 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 $FeCl_3$) in polyethylene tanks supplemented with nettle leaves, commercial fish food and cellulose tissue. Cultures were maintained under constant aeration at 20 ± 1 °C and under light:dark cycle of 16:8.

2.3. Treatments

C. riparius egg masses were taken from the laboratory population after oviposition, placed in 6-well culture plates containing culture medium and maintained under standard conditions for 24 h prior to testing. Each egg mass was divided into two; one half was submitted to the experimental treatment and the other one used for control. For gene expression analysis, embryos were exposed to 4×10^{-6} M benzophenone-3 diluted in culture medium for different experimental times (12, 24, 36 h). For developmental studies, the eggs masses were divided into two after oviposition, and treated either with BP-3 (4×10^{-6} M) or maintained BP-3-free in the same medium until hatching to analyze the timing of embryo development. Samples were maintained in the dark and the medium was renewed every 24 h. Non-treated control embryos were exposed to the same concentration of solvent (ethanol 0.1%) as the corresponding treatment. The percentage of hatching was obtained counting the number of eggs hatched per total number of eggs. Three replicates were carried out for each treatment and each one was also measured in triplicate.

Salivary glands from fourth instar larvae (5th phase) were dissected and rinsed for fifteen minutes with Schneider's Insect Medium (Sigma–Aldrich) before starting the test. From each animal, one of the pair of glands was incubated in insect medium containing the experimental treatment and the other in the same conditions, including solvent, but treatment free for 30, 60 and 180 min. In each experiment, five animals were selected and, therefore, five glands were exposed either to benzophenone-3 (4×10^{-7} M) or to 20-hydroxyecdysone (2×10^{-7} M), while the corresponding five partners were used as controls exposed to the solvent (ethanol 0.2%). Three independent experiments were performed for each analysis and time point, each one with three experimental replicates.

2.4. RNA isolation and cDNA synthesis

To isolate embryo RNA, a prior treatment was performed to remove the gelatinous cover of the egg mass. Briefly, the egg mass was treated with 1× PBS (137 mM ClNa, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and 0.2% sodium hypochlorite until the gelatinous cover disappeared and the eggs reached the bottom of the microcentrifuge tube. Subsequently, the eggs were washed several times with 1× PBS until the sodium hypochlorite was completely eliminated. Total RNA was extracted from salivary glands and embryos using TRIzol Reagent (Invitrogen) following the manufacturer's protocol. The samples were then treated with RNase-free DNase (Roche) for 90 min and an organic extraction with phenol-chloroform was carried out. Finally, RNA was precipitated by isopropyl alcohol (0.5 v/v), washed with 70% ethanol, and resuspended in DEPC water. The concentration and purity of the isolated RNA were checked by absorption spectroscopy (Biophotomer Eppendorf). Purified RNA was stored at −80 °C. Aliquots of 0.5 μg of total RNA were reverse-transcribed into cDNA using 100 units of M-MLV enzyme (Invitrogen), in the presence of 0.5 μg oligo dT_{20} primer (Sigma) and 0.5 mM dNTPs (Biotools) at 37 °C for 50 min in a reaction volume of 20 μl.

2.5. Real Time RT-PCR

The cDNA obtained was utilized to amplify the sequence of interest by Quantitative Real Time PCR (qRT-PCR) using a CFX96 thermocycler (BioRad). The mRNA expression profile of the *EcR*, *usp*, *E74* and *hsp70* genes in control and under different treatments was performed with 25 ng of template cDNA, 0.3 μM of forward and reverse primers and SsoFast EvaGreen Supermix (BioRad) in a final volume of 20 μl. *Actin-ß* and *glyceraldehyde 3-phosphate dehydrogenase* (*GAPDH*) were employed as endogenous reference genes (Martínez-Guitarte et al., 2012). Ec*R* and *usp* specific primers were designed from a partial clone obtained by PCR (Planelló et al., 2008, 2010). The *hsp70* primers were designed from *Chironomus riparius* sequences