Aquatic Toxicology 140–141 (2013) 19–26

Contents lists available at SciVerse ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

No substantial changes in estrogen receptor and estrogen-related receptor orthologue gene transcription in *Marisa cornuarietis* exposed to estrogenic chemicals☆,☆☆

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ARTICLE INFO

Article history:
Received 29 January 2013
Received in revised form 1 May 2013
Accepted 3 May 2013

Keywords:
Mollusc
Estrogen receptor
Estrogen-related receptor
Gene transcription
Estrogen
Exposure

ABSTRACT

Estrogen receptor orthologues in molluscs may be targets for endocrine disruptors, although mechanistic evidence is lacking. Molluscs are reported to be highly susceptible to effects caused by very low concentrations of environmental estrogens which, if substantiated, would have a major impact on the risk assessment of many chemicals. The present paper describes the most thorough evaluation to-date of the susceptibility of *Marisa cornuarietis* ER and ERR gene transcription to modulation by vertebrate estrogens *in vivo* and *in vitro*. We investigated the effects of estradiol-17β and 4-*tert*-Octylphenol exposure on *in vivo* estrogen receptor (ER) and estrogen-related receptor (ERR) gene transcription in the reproductive and neural tissues of the gastropod snail *M. cornuarietis* over a 12-week period. There was no significant effect ($p > 0.05$) of treatment on gene transcription levels between exposed and non-exposed snails. Absence of a direct interaction of estradiol-17β and 4-*tert*-Octylphenol with mollusc ER and ERR protein was also supported by *in vitro* studies in transfected HEK-293 cells. Additional *in vitro* studies with a selection of other potential ligands (including methyl-testosterone, 17α-ethinylestradiol, 4-hydroxytamoxifen, diethylstilbestrol, cyproterone acetate and ICI182780) showed no interaction when tested using this assay. In repeated *in vitro* tests, however, genistein (with mcER-like) and bisphenol-A (with mcERR) increased reporter gene expression at high concentrations only ($>10^{-6}$ M for Gen and $>10^{-5}$ M for BPA, respectively). Like vertebrate estrogen receptors, the mollusc ER protein bound to the consensus vertebrate estrogen-response element (ERE). Together, these data provide no substantial evidence that mcER-like and mcERR activation and transcript levels in tissues are modulated by the vertebrate estrogen estradiol-17β or 4-*tert*-Octylphenol *in vivo*, or that other ligands of vertebrate ERs and ERRs (with the possible exception of genistein and bisphenol A, respectively) would do otherwise.

1. Introduction

The susceptibility of molluscs to morphological and physiological disruption by estrogenic compounds is a subject of current debate. Amongst the most extreme effects reported are those exerted by bisphenol A (BPA), 4-*tert* octylphenol (4-*t*-OP) and 17α-ethinylestradiol (EE2) on reproductive output and morphology of the neo-tropical freshwater snail *Marisa cornuarietis* (Oehlmann et al., 2000, 2006; Schulte-Oehlmann et al., 2004). These published reports indicate that this species is extremely sensitive to very low concentrations of these compounds. The effects include increased oocyte production and egg-laying in females and gross morphological effects on the sex organs in both developing juveniles (*e.g.* formation of additional sex organs in females) and adults (*e.g.* reduction in male penis length). In direct conflict with these reports are those in which adult *M. cornuarietis* were exposed to BPA using a different experimental design to those employed in (Oehlmann et al., 2000) and (Oehlmann et al., 2006) showing clearly that these effects were not observed (Forbes et al., 2007). These conflicting reports have fuelled controversy (Dietrich et al., 2006) surrounding the true sensitivity of this species, and

☆ This work was funded by BBSRC grant 100/S183300.

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http://dx.doi.org/10.1016/j.aquatox.2013.05.002

molluscs in general, to estrogen mimics and the perceived safety of the aquatic environment from the impacts of these xenoestrogens. BPA, in particular, is purported to be much more potent in molluscs than in other aquatic organisms (Oehlmann et al., 2006).

Perhaps the most striking feature of the effects reported (Oehlmann et al., 2000, 2006; Schulte-Oehlmann et al., 2004) is their resemblance to those that occur in vertebrate species in response to xenoestrogen exposure (Jobling et al., 2004). This may indicate that one or more estrogenic mechanisms have been conserved through evolution and are present in both molluscs and vertebrates. In vertebrates, the classically understood mechanism of estrogen action is *via* estrogen receptors (ERs). There are two main ER subtypes, ERα (Green et al., 1986) and ERβ (Kuiper et al., 1996). Lipophilic estrogens can enter cells *via* passive diffusion and are subsequently bound by ERs. This binding results in a conformational change in the ER protein, which then binds to specific DNA recognition sequences in the promoter regions of various genes and can regulate their transcription (Kimbrel and McDonnell, 2003). Published studies show that ER orthologues have been identified in several species of mollusc (Thornton et al., 2003; Keay et al., 2006; Kajiwara et al., 2006). However, all of these studies show lack of binding of ER protein to vertebrate estrogens or xenoestrogens *in vitro* using reporter assays, and endogenous ligands have not been found. Our laboratory published the cDNA cloning of a molluscan orthologue of vertebrate ERs from *M. cornuarietis* (Bannister et al., 2007). One of these factors, mcER-like, is highly similar to vertebrate ERs in terms of sequence similarity and structure; however, unlike its vertebrate orthologues (Routledge et al., 2000), it does not bind 17β-estradiol (E2). A second factor was cloned with high sequence identity to vertebrate (Giguere et al., 1988) and insect (Östberg et al., 2003) estrogen receptor-related receptors (ERRs). Like mcER-like and vertebrate and invertebrate ERRs, mcERR protein did not bind E2 in radio-ligand binding trials. Indirect evidence of ER-mediated estrogen binding is provided by Oehlmann and colleagues (Oehlmann et al., 2006), who observed that *in vivo* co-exposures of *M. cornuarietis* to BPA and classical anti-estrogens (Tamoxifen and Faslodex) negated the responses attributed to BPA. In the same study, it was shown that tritiated E2 could be displaced from cytosolic extracts of *M. cornuarietis* tissues by unlabelled E2. None of the above work demonstrates specific binding to, or activation of, classical estrogen-signalling pathway factors *in vivo*. This is necessary in order to prove that steroid estrogens and endocrine disrupting compounds interact with mollusc reproductive systems through interaction with the ER in *M. cornuarietis* and other species of mollusc. Here we test the hypothesis that the reported sensitivity of *M. cornuarietis* to estrogenic chemicals is mediated by the mollusc ER and/or ERR by examining the transcriptional responses of these genes during a 12-week exposure to a natural vertebrate steroid estrogen (E2) or a xenoestrogen (4-*t*-OP). We also assess the ability of 4-*t*-OP, E2 and a range of other natural and synthetic estrogenic chemicals (known to interact with estrogen receptors in vertebrates) for their ability to mediate reporter gene expression in a 2-hybrid system comprising HEK-293 cells transfected with mcER-like and mcERR ligand-binding domains coupled to the GAL4-DNA-binding domain and the VP16-transactivation domain.

2. Materials and methods

2.1. Chemicals

17β-Estradiol (E2; ≥98% pure), 17α-ethinylestradiol (EE2; ≥98% pure), genistein (GEN; ≥98%), diethylstilbestrol, (DES; ≥99%), bisphenol A (BPA; 97%), cyproterone acetate (CPA; ≥98%), methyltestosterone (MT; ≥97%), hydroxy-tamoxifen (Tam-OH; ≥98%), ICI 182,780 (≥98%), and 4-*tert*-octylphenol (4-*t*-OP; 97%) were purchased from Sigma (Dorset, UK) and were research grade chemicals. Stocks of E2 and 4-*t*-OP for use in the *in vivo* exposure were prepared in methanol.

2.2. Animals and husbandry

M. cornuarietis were obtained from stocks bred at Brunel University, UK, which were originally derived from a stock maintained by Prof. Jörg Oehlmann's laboratory (Johann Wolfgang Goethe University, Frankfurt-am-Main, Department of Ecology & Evolution, Frankfurt-am-Main, Germany). Snails were maintained under static conditions in large 60 L static glass tanks at 22 °C and a photoperiod of 12l:12d and fed with organic lettuce. The exposures were carried out at AstraZeneca's Brixham Environmental Laboratory.

2.3. In vivo exposure to E2 and 4-t-OP experimental design

Three replicate groups of 20 adult snails between 6 and 8 months old (each containing 10 male and 10 female snails based on morphological examination) were exposed to dilution water control, a solvent control, E2 (at 10, 100 and 1000 ng/L) and 4-*t*-OP (5 and 25 μg/L) in flow through aquaria (volume 20 L) at 22 °C with a 12-h light/dark cycle and 0.025 g Tetramin flake food (Tetra) per snail per day (5 days per week). All flake food is tested for absence of estrogenicity using the yeast estrogen screen assay (Routledge and Sumpter, 1996) prior to use. Water samples were also taken for determination of E2 levels using the Yeast Estrogen Screen (Routledge and Sumpter, 1996) and 4-*t*-OP by HPLC analysis conducted at AstraZeneca. Samples were taken 1 week before exposure and throughout the 12 week exposure (approximately 3, 9 and 12 weeks after exposure) to ensure that the dosing into the water produced concentrations close to nominal values. Sampling of the snails (2 male and 2 females per tank selected at random to obtain 6 snails of each sex per treatment) took place 24 h prior to exposure, and after one week, six weeks and 12 weeks of exposure. Each snail was weighed and measured (shell height and aperture width) and tissues (ganglia, gonad-digestive complex, male penis and sheath, female albumin glands) were dissected out and snap frozen on liquid nitrogen for RNA extractions. Absolute quantitative real-time PCR (QPCR) was carried out to measure mcER-like and mcERR-like mRNA transcript abundance in each of the tissues in each snail as described previously (Bannister et al., 2007). Reproductive output in terms of numbers of eggs and egg-masses produced was recorded at 2-week intervals throughout the study (including the two-week baseline period).

2.4. Quantitative real-time PCR (QPCR)

Total RNA was isolated from individual organs of replicate individuals, using Tri-Reagent (Sigma), according to the manufacturer's recommended protocol. All total RNA samples were treated with *DNase* I (Invitrogen) to remove traces of genomic DNA. To determine absolute amounts of transcripts, RNA standards were synthesised *in vitro* from DNA templates, and Absolute Quantitative Real-time PCR was carried out using the one-step QuantiTect SYBR Green RT-PCR kit (Qiagen) with an ABI PRISM 7900 Sequence Detection System (Applied Biosystems) as described previously (Bannister et al., 2007). Each experiment included absolute negative controls (no template), no reverse transcriptase controls, negative tissue controls (RNA isolated from fathead minnow testis) and internal reference tissue controls (non-exposed *M. cornuarietis* RNA preparation). To account for inter-assay variability between different qPCR plates, a positive control (RNA prepared from non-exposed female *M. cornuarietis* albumin gland) was included in each assay plate. The qPCR data presented here are expressed as