Aquatic Toxicology 90 (2008) 261–268

Contents lists available at ScienceDirect

Aquatic Toxicology

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

Effects of synthetic polycyclic musks on estrogen receptor, vitellogenin, pregnane X receptor, and cytochrome P450 3A gene expression in the livers of male medaka (*Oryzias latipes*)

Ryoko Yamauchi[a], Hiroshi Ishibashi[a,b,*], Masashi Hirano[a], Taiki Mori[a], Joon-Woo Kim[a], Koji Arizono[a]

[a] *Faculty of Environmental and Symbiotic Sciences, Prefectural University of Kumamoto, 3-1-100 Tsukide, Kumamoto 862-8502, Japan*
[b] *Center for Marine Environmental Studies (CMES), Ehime University, Bunkyo-cho 2-5, Matsuyama 790-8577, Japan*

ARTICLE INFO

Article history:
Received 4 August 2008
Received in revised form
10 September 2008
Accepted 11 September 2008

Keywords:
Polycyclic musk fragrance
Oryzias latipes
Estrogen receptor α
Vitellogenin
Pregnane X receptor
Cytochrome P450 3A

ABSTRACT

This study demonstrates the effects of synthetic polycyclic musks such as Galaxolide (HHCB), Tonalide (AHTN), Traseolide (ATII), Celestolide (ADBI), Phantolide (AHMI) and Cashmeran (DPMI), both on the early life stage and on gene expression in the livers of male medaka (*Oryzias latipes*). The toxicity ranking (the 96-h median lethal concentration) of the chemicals tested on 24-h-old medaka larvae descended in the order HHCB (0.95 mg/L) = ATII (0.95 mg/L) > AHTN (1.0 mg/L) > AHMI (1.2 mg/L) > ADBI (2.0 mg/L) ≫ DPMI (12 mg/L), indicating high acute toxicity of these compounds on the early life stages of medaka. Expression analysis of hepatic vitellogenin (VTG) protein showed potential estrogenic effects upon the addition of AHTN and HHCB, indicative of the induction of VTG synthesis in the livers of male medaka. We also investigated mRNA expression levels of two estrogen receptor (ER) subtypes (ERα and β), two VTGs (VTG I and II), pregnane X receptor (PXR), and two cytochromes P450 (CYP) 3As (CYP3A38 and 3A40) in the livers of male medaka treated with AHTN and HHCB at 5, 50 and 500 μg/L. Quantitative real-time PCR analyses revealed that hepatic ERα, VTG I, VTG II, and CYP3A40 mRNA responded to 500 μg/L of AHTN and/or HHCB after 3 days exposure, whereas no effects of these compounds on ERβ, PXR, and CYP3A38 mRNA transcription were observed. These results suggest that certain polycyclic musks, including AHTN and HHCB, induce the expression levels of hepatic ERα and VTG mRNA/protein and modulate expression levels of CYP3A40 mRNA in the livers of male medaka.

1. Introduction

Synthetic polycyclic musks, such as 7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene (AHTN, Tonalide) and 1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta[γ]-2-benzopyran (HHCB, Galaxolide), are widely used as fragrance compounds in a variety of personal care products including detergents and cosmetics (Van de Plassche and Balk, 1997; Rimkus, 1999). Bioaccumulation and widespread distribution of these compounds have been reported in humans and wildlife (Nakata et al., 2006; Kannan et al., 2005). Previous studies have demonstrated the occurrence of AHTN and HHCB in various environmental samples from rivers, oceans, surface water, sewage sludge and sediment (Rimkus, 1999; Aaron and Keri, 2004; Bester et al., 1998; Boxall et al., 2004; Daughton and Ternes, 1999; Draisci et al., 1998; Fromme et al., 2001, 1999; Gatermann et al., 1999; Heberer et al., 1999; Kolpin et al., 2002). Recent surveys have also reported the occurrence of various polycyclic musks, such as Traseolide (ATII), Celestolide (ADBI), Phantolide (AHMI), and Cashmeran (DPMI), in the aquatic environment (Shek et al., 2008; Zeng et al., 2007). While the concentrations of these polycyclic musks in surface water are in the ng to μg/L range, in fish, levels from 10 to 10,000 μg/kg fat have been found (Rimkus, 1999). These studies indicate the importance of investigating the potential biological effects and risks of polycyclic musks in aquatic species, including fish.

Due to the high bioaccumulation potential of polycyclic musks in the environment, their potential toxic effects on the biota are of great concern (Tanabe, 2005). Various *in vitro* reporter gene assays have indicated potential estrogenic and antiestrogenic effects of polycyclic musks including AHTN, HHCB, ATII, ADBI, AHMI and DPMI towards human estrogen receptor (ER) (Seinen et al., 1999; Schreurs et al., 2005a,b; Mori et al., 2007). On the other hand, an *in vivo* mouse uterotrophic assay showed that AHTN (15 and 50 ppm)

* Corresponding author at: Center for Marine Environmental Studies (CMES), Ehime University, Bunkyo-cho 2-5, Matsuyama 790-8577, Japan.
Tel.: +81 89 927 8194; fax: +81 89 927 8194.
E-mail address: hiroishi@agr.ehime-u.ac.jp (H. Ishibashi).

0166-445X/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquatox.2008.09.007

or HHCB (50 and 300 ppm) added to the diet of female juvenile mice for 2 weeks did not affect the weight of the thymus or uterus, indicating no estrogenic effects of these polycyclic musks *in vivo* (Seinen et al., 1999). Similarly, an *in vivo* study using a transgenic zebrafish assay did not find any evidence for estrogenic effects of AHTN and HHCB (Schreurs et al., 2004). However, this study also demonstrated that estradiol-17β (E2)-induced transactivation is suppressed by treatment with AHTN or HHCB, indicating antiestrogenic effects of these compounds *in vivo* (Schreurs et al., 2004). Taken together, data currently available are ambiguous regarding the estrogenic effects of AHTN and HHCB *in vivo*.

Vitellogenin (VTG), an estrogen-inducible phosphoprotein and a complex precursor protein of egg yolk, is a useful biomarker for evaluating estrogenic compounds in aquatic environments (Sumpter and Jobling, 1995). A number of environmental estrogens, including synthetic estrogens and alkylphenolic compounds, induce VTG synthesis in the livers of male medaka (Ishibashi et al., 2004, 2005, 2006). These environmental estrogens, including endogenous E2, regulate estrogen-responsive genes such as VTG by binding to a specific ER; the estrogen–ER complex then interacts with the estrogen-responsive elements (EREs) of the target promoter genes to modulate their transcriptional activity. Our previous studies demonstrated that certain estrogenic compounds, such as E2, nonylphenol (NP), bisphenol A (BPA), and fluorotelomer alcohols (FTOHs), induce the expression levels of hepatic VTG mRNA mediated by the ERα signaling pathway in male medaka (Ishibashi et al., 2008; Yamaguchi et al., 2005).

The pregnane X receptor (PXR), a member of the orphan nuclear receptor superfamily, is activated by a wide variety of endogenous substances such as steroid hormones, bile acids and xenobiotics (Kliewer et al., 1998; Blumberg et al., 1998; Moore et al., 2002). In mammals, many xenobiotics induce the expression of cytochrome P450 (CYP) 2B/3A via the PXR signaling pathway (Maglich et al., 2002; Bertilsson et al., 1998). Only one recent study has demonstrated transcriptional activation of human PXR by treatment with the polycyclic musk HHCB, using an *in vitro* transiently transfected reporter gene assay (Mnif et al., 2007). This result suggests that modulation of typical CYP2B/3A expression may affect steroid hormone metabolism. To date, PXR has been cloned and characterized in mammals (Moore et al., 2002), but little is known about its molecular characterization (Maglich et al., 2003; Moore et al., 2002), or the signaling modulation via PXR activated by environmental contaminants in non-mammalian species such as teleost fish (Mortensen and Arukwe, 2006; Meucci and Arukwe, 2006).

Medaka (*Oryzias latipes*) is widely used in ecotoxicology and has been proposed to be a suitable fish for evaluating environmental chemicals. Early life stage medaka are considered to be sensitive biosensors in toxicity tests. In this study, we used medaka as a test organism, and investigated the effect of six polycyclic musks AHTN, HHCB, ATII, ADBI, AHMI and DPMI on its early life stage. Furthermore, in order to evaluate the potential estrogenic effects of major polycyclic musks AHTN and HHCB *in vivo,* we investigated the expression levels of estrogen-responsive genes such as ERs (ERα and β) and VTGs (VTG I and II), as well as the level of VTG protein, in the livers of male medaka exposed to polycyclic musks in their aqueous environment. We also investigated the effects of polycyclic musks on the PXR-CYP3As signaling pathway.

2. Materials and methods

2.1. Test chemicals

The polycyclic musks AHTN, HHCB, ATII, ADBI, AHMI and DPMI were obtained from Promochem (Teddington, UK). E2 (a positive control for hepatic VTG expression analysis) was obtained from Sigma Chemical Industries, Ltd., Tokyo, Japan. Stock solutions of the test compounds were prepared by dissolving the reagents in DMSO (dimethyl sulfoxide, Wako Pure Chemical Industries, Ltd., Tokyo, Japan). Test solutions were prepared by adding stock solutions to water.

2.2. Acute toxicity test for medaka larvae

For each treatment, fifteen 24-h-old larvae were placed in glass beakers containing 100 mL of each test solution at 25 ± 1 °C with a 16:8 h light:dark photoperiod cycle following the procedure described previously (Ishibashi et al., 2005), and were not fed during the toxicity tests. Each group of 24-h-old larvae was exposed to polycyclic musk concentrations (HHCB: 0.60–1.5 mg/L, AHTN: 0.53–1.3 mg/L, ATII: 0.48–1.2 mg/L, ADBI: 1.3–3.3 mg/L, AHMI: 0.76–1.9 mg/L, DPMI: 8.7–18 mg/L) prepared by diluting the stock solutions in dechlorinated tap water. Each treatment was conducted at 25 ± 1 °C for 96 h. The controls were exposed to solvent carrier only (0.1% DMSO). During the experimental period, the test solutions in the beakers were changed daily. The larvae were observed daily under a stereoscopic microscope, and dead larvae, characterized by the absence of heart activity, were removed daily. The 96-h median lethal concentration (LC_{50}) values for the 24-h-old larvae were calculated by probit analysis.

2.3. Exposure of male medaka to polycyclic musks

The medaka fish used in this study have been maintained for several years in glass tanks in our laboratory. In the pre-exposure period, about 100 male medaka were selected and kept in a 30-L glass tank under a 16:8 h light:dark photoperiod at 25 ± 1 °C for 2 weeks. In order to eliminate the effects of dietary phytoestrogen on VTG synthesis, these male fish were fed an estrogen-free diet of *Artemia nauplii* (<24 h after hatching) (Inudo et al., 2004) twice daily.

The male medaka were exposed to the test compounds according to the method previously reported (Ishibashi et al., 2008); the concentrations used in these exposure experiments were based on the results from the 96-h acute toxicity test for polycyclic musks. Three (for VTG protein analysis) or six (for gene expression analysis) adult male medaka (about 4 months after hatching) were exposed to test chemicals in 1-L glass beakers for 3 days at 25 ± 1 °C. Test solutions were changed daily during the exposure period, and the fish were subjected to a 16:8 h light:dark photoperiod. The fish were fed a diet of *A. nauplii* (<24 h after hatching) twice daily for 3 days. The control group was only exposed to the carrier solvent (0.01% DMSO), and E2-treated control group was exposed to the nominal concentration of 1 nM of E2. At the end of the exposure period, the livers were sampled, rapidly frozen in liquid nitrogen, and stored at −80 °C until analysis.

2.4. Determination of AHTN and HHCB concentrations in test water

AHTN and HHCB were analyzed following the method described elsewhere (Reiner and Kannan, 2006) with some modifications. The AHTN and HHCB concentrations in the test water of each treatment group were measured once a day during the exposure period. Fifty milliliters of hexane, 20 mL of methanol, and 100 ng of phenanthrene-d_{10} (used as an internal standard) were added to each of the test water samples (500 mL); then, AHTN and HHCB were extracted by shaking at 350 rpm for 60 min. The hexane layer was removed, and the samples were re-extracted with 50 mL of hexane. The combined hexane extract was evapo-