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Sensitization of vitellogenin gene expression by low doses of octylphenol is mediated by estrogen receptor autoregulation in the *Bombina orientalis* (Boulenger) male liver

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ABSTRACT

This study aimed to elucidate the mechanisms by which alkylphenols disrupt endocrine function in wild amphibians in Korea. To this end, the effects of 4-*tert*-octylphenol (OP), 17β-estradiol (E_2), and estrogen receptor (ER) agonists on the expression profiles of vitellogenin (VTG) and ERs were examined in livers obtained from male *Bombina orientalis* toads. A single injection of E_2 (10 μg/kg; 0.03 μmol/kg) induced transcription of VTG mRNA at 2 days post injection; however, injection of either the ERα-selective agonist propyl-(1H)-pyrazole-1,3,5-triyl-trisphenol (PPT, 50 μg/kg; 0.12 μmol/kg) or the ERβ-selective agonist 2,3-bis-(4-hydroxyphenyl)-propionitrile (DPN, 50 μg/kg; 0.20 μmol/kg) did not affect the expression of VTG. This finding suggests that both ERα and ERβ are required to induce transcription of VTG in the male *B. orientalis* liver. Interestingly, E_2, PPT, and DPN induced transcription of ERα, which was also reflected on the protein level; however, these alkylphenols did not affect ERβ transcription. Similarly, VTG transcription was induced by a single injection of 1–100 mg/kg (0.04–484.66 μmol/kg) OP, while 0.1 mg/kg (0.48 μmol/kg) OP had no effect on VTG transcription. This result suggests that the lowest observable effect concentration (LOEC) of OP for induction of VTG transcription in the male liver is 1 mg/kg (4.84 μmol/kg). Furthermore, treatment with E_2 (10 μg/kg; 0.03 μmol/kg) or OP (1 mg/kg; 4.84 μmol/kg) significantly upregulated ERα transcription, and a 10 mg/kg (48.46 μmol/kg) dose of OP significantly upregulated ERβ transcription. The ER antagonist ICI 182,780 decreased the basal levels of ERα and ERβ mRNA, and also prevented E_2-mediated and OP-mediated induction of VTG, ERα, and ERβ transcription. A second injection of 0.1 mg/kg (0.48 μmol/kg) OP after a two-day interval significantly upregulated the transcription of VTG and ERα, but not of ERβ. These results suggest that sensitization of VTG transcription by repeated exposure to OP is mediated by the induction of ERα. Different combinations of alkylphenols that are ubiquitous in the freshwater system in Korea could potentially exert a synergistic effect on endocrine disruption. Thus, chronic exposure to alkylphenols, even at their NOECs, could still disrupt endocrine function in *B. orientalis*.

1. Introduction

Endocrine disruption is believed to be a contributing factor to the global decline of amphibians (Renner, 2002). Natural and synthetic estrogens enter water systems from anthropogenic sources via sewage treatment effluent (Desbrow et al., 1998), discharge from the industrial manufacturing of pharmaceuticals (Heberer, 2002), and agricultural runoff, which often contains animal manure that is used as fertilizer (Hanselman et al., 2003). The observation of various abnormalities, such as the feminization of wild animals, implies that water contamination with estrogenic compounds is an undeniable concern (Vos et al., 2000; Hutchinson et al., 2000). In amphibians, xenoestrogens induce the expression of estrogen-responsive genes, which have been associated with adverse effects on amphibian development (Kloas et al., 1999; Lutz and Kloas, 1999; Mosconi et al., 2002; Mackenzie et al., 2003; Ahn et al., 2011). Alkylphenolic compounds, which are used in a variety of human commercial products, are known to be estrogenic in vertebrates (Ying et al., 2002; Waring and Harris, 2005). Among these alkylphenols, 4-*tert*-octylphenol (OP) is one of the two main degradation products of alkylphenol polyethoxylates. These compounds are nonionic surfactants that are often used in household and industrial detergents (Nimrod and Benson, 1996); moreover, OP accounts for about 15% of all commercial alkylphenols in the USA and Canada (Bennett and Metcalfe, 1998). Although nonylphenol (NP), another degradation product of alkylphenol

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polyethoxylates, is the predominant contaminant in most water systems, the estrogenic potency of OP has been shown to be higher than that of NP (White et al., 1994). In Korean rivers, OP has been detected at concentrations ranging from 2.24 to 16.78 ng/L (10.86–81.33 nM) in surface water, and at 0.5 ng/L (2.42 nM) in the effluent from sewage treatment plants (Duong et al., 2010; Ra et al., 2011). Thus, OP in the aquatic environment may exert toxic effects on amphibians during their embryonic, larval, and adult life cycle stages. Due to its structural similarity to estrogen, OP can induce the transcription of estrogen-responsive genes and thus exert adverse effects on the embryonic development, sexual behavior, and sexual differentiation of amphibians (Nishimura et al., 1997; Kloas et al., 1999; Lutz and Kloas, 1999; Mayer et al., 2003; Huang et al., 2005; Porter et al., 2011).

Vitellogenin (VTG) is an egg yolk precursor that is synthesized in the livers of female oviparous and ovoviviparous vertebrates (Wahli et al., 1981; Wallace, 1985). In amphibians, several natural estrogens and xenoestrogens have been shown to upregulate VTG transcription and protein production, both in male livers and in hepatocyte cultures (Wangh and Knowland, 1975; Carnevali et al., 1995; Bögi et al., 2003; Rotchell and Ostrander, 2003; Gye and Kim, 2005; Kang et al., 2006). Therefore, VTG induction in males has been used as a biomarker for measuring the estrogenicity of certain chemicals and environmental media (Sumpter and Jobling, 1995). The two estrogen receptors, ERα and ERβ, mediate the estrogenic effects of natural estrogens and xenoestrogens in both humans and wild animals (Kuiper et al., 1998; Rotchell and Ostrander, 2003; Melzer et al., 2011). In *Xenopus*, OP binds directly to ERs and activates the VTG promoter (Huang et al., 2005). Moreover, estrogens and xenoestrogens have also been reported to affect the expression of VTG, ERα, and ERβ in fishes (Soverchia et al., 2005; Nagler et al., 2010). However, in amphibians, the transcriptional responses of ERα and ERβ to natural estrogens and xenoestrogens have not yet been investigated. An assessment of the ecological effects of endocrine disruptors on freshwater fishes and frogs in Korea revealed that the concentration of OP in the muscle tissue of adult bullfrogs (*Rana catesbeiana*) was 10 times higher than that in fishes (NIER, 2001). Moreover, the plasma VTG levels of male bullfrogs were similar to those of females in the surveyed areas (NIER, 2003). In the present study, we examined the effects of OP on ERα, ERβ, and VTG expression. As a model organism, we selected the male fire-bellied toad, *Bombina orientalis*, which is a common native Korean anuran. Importantly, *B. orientalis* can be induced via gonadotropin to ovulate and spawn eggs at three-month intervals in the laboratory, thus permitting developmental toxicity assessment on embryos year-round (Park et al., 2010). Moreover, the sequence of *B. orientalis* ERα is available (GenBank accession no. FJ387577.1), and a homologue of VTG A2 has been identified (Lee and Gye, 2004). Furthermore, a protocol for the quantitative analysis of hepatic VTG mRNA has been established (Gye and Kim, 2005; Kang et al., 2006). In an effort to understand the mechanisms of endocrine disruption by estrogenic alkylphenols in a native Korean toad species, we examined the effects of OP on the expression of VTG, ERα, and ERβ in livers from adult male *B. orientalis*. We also tested the hypothesis that autoregulation of ERα and ERβ in the male liver by OP may mediate VTG induction after chronic exposure to low doses of xenoestrogens.

2. Materials and methods

2.1. Animals

Adult *B. orientalis* were bred and reared in the Hanyang University Aquarium. The toads were maintained on a diurnal 14 h light and 10 h dark cycle at 20–22 °C, and were fed crickets and mealworms three times a week. Adult toads with a mean body weight (BW) of 8.0 ± 0.5 g were used for all experiments. All experiments conducted on amphibians followed the procedures outlined in the "Guidelines for the Use of Live Amphibians and Reptiles in Field and Laboratory Research" publication (ASIH, 2004) (http://www.asih.org/files/hacc-final.pdf). As a control for ERα and ERβ in Western blots, mouse uterus tissue was obtained from an adult female mouse that had been euthanized after asphyxiation in CO_2. All mouse procedures were approved by the Institutional Animal Care and Use Committee of Hanyang University (HY-IACUC-11-044).

2.2. Chemical exposure

The 17β-estradiol (E2) (MW = 272.38; Sigma Aldrich, St. Louis, MO), 4-tert-octylphenol (OP; MW = 206.33; Aldrich Chemical Co., Milwaukee, WI), ICI 182,780 (ICI; MW = 606.77; Sigma Aldrich) as an ERs antagonist, and ER subtype specific agonists, propyl pyrazole triol (PPT; MW = 386.44; Abcam, Cambridge, MA) estrogenic compounds were used for experiments involving ERα and diarylpropionitrile (DPN; MW = 239.27; Abcam) was used for experiments involving ERβ. Selected adult male toads received an intraperitoneal injection of OP (0.01, 0.1, 1, 10, or 100 mg/kg BW; 0.04, 0.48, 4.84, 48.46, or 484.66 μmol/kg BW) or E_2 (10 μg/kg; 0.03 μmol/kg) dissolved in sesame oil. These doses were found to effectively induce VTG expression in *B. orientalis* in initial optimization experiments. For some experiments, toads were given 50 μg/kg (0.12 μmol/kg) PPT and 50 μg/kg (0.20 μmol/kg) DPN. The doses of PPT and DPN were determined according to previous studies (Frasor et al., 2003; Neese et al., 2010). ICI was given at 1 mg/kg (1.64 μmol/kg). This dose was based on the binding affinity of ICI to ERs, which is 100-fold lower than that of E_2 (Preisler-Mashek et al., 2002). ICI was given either alone or in combination with E_2, OP, PPT, or DPN. As a vehicle control (VC), males were given sesame oil only. The total injection volume was 100 μL per animal. To test the effects of repeated exposure to OP on gene expression in the male liver, two OP injections were given with a two-day interval. The concentration of OP used (0.1 mg/kg; 0.48 μmol/kg) was below the lowest observed effect concentration (LOEC). Toads were returned to the aquaria and sampled 48 h after the second injection. Five male toads were used in each experimental group.

2.3. Degenerate PCR and cloning of ERβ cDNA

Female toads were euthanized by inhalation of ether to minimize pain, and their livers were removed. Total liver RNA was isolated using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) according to the manufacturer's instructions. The concentrations of all RNA preparations were determined prior to storage at −85 °C until use. For cDNA synthesis, RNA (1 μg) was reverse transcribed for 60 min at 42 °C in a 20 μL reaction with 50 units of MuLV reverse transcriptase and 2.5 μM oligo d(T)16 primer, according to the manufacturer's standard protocol (Applied Biosystems, Foster City, CA). Degenerate PCR was performed with degenerate primers for the ERβ cDNA sequence, which was based on multiple anuran ERβ mRNA sequences (*Xenopus laevis*, NM_001130954.1; *Xenopus tropicalis*, NM_001040012.1; *Rana rugosa*, FJ828859.1; *Bufo rangeri*, AB524915.1) (Table 1). PCR products were ligated into the pGEM-T Easy vector (Promega, Madison, WI), and the resultant constructs were transformed into DH5α competent cells. More than 10 positive colonies were selected by blue/white screening. DNA sequencing was performed using M13 primers.