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Application of a yeast estrogen screen in non-biomarker species *Varicorhinus barbatulus* fish with two estrogen receptor subtypes to assess xenoestrogens

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Abstract

Xenoestrogens can interfere with normal estrogen signaling by competitively binding to the estrogen receptor (ER) and activating transcription of target genes. In this study, we cloned the estrogen receptor alpha (*vb*ERα) and beta 2 (*vb*ERβ2) genes from liver of the indigenous Taiwanese cyprinid fish *Varicorhinus barbatulus* and tested the direct impact of several xenoestrogens on these ERs. Transcriptional activity of xenoestrogens was measured by the enzymatic activity of estrogen responsive element (ERE)-containing β-galactosidase in a yeast reporter system. The xenoestrogens tested were phenol derivatives, DDT-related substances, phthalic acid esters, and polychlorinated biphenyls, with 17β-estradiol (E2) as a subjective standard. The phenol derivatives [4-nonylphenol (4-NP), 4-*t*-octylphenol (4-*t*-OP) and bisphenol A (BPA)] exhibited significant dose-dependent responses in both ligand potency and ligand efficiency. Consistent with yeast assays using human or rainbow trout ERs, we observed a general subtype preference in that *vb*ERα displayed higher relative potencies and efficiencies than *vb*ERβ2, although our assays induced a stronger response for xenoestrogens than did human or trout ERs. Whereas 4-NP and 4-*t*-OP have similar EC50 values relative to E2 for both ER subtypes, the strong estrogenic response of BPA markedly differentiates *vb*ERα from *vb*ERβ2, suggesting possible species-specific BPA sensitivity. We report that the ameliorative yeast tool is readily applicable for indigenous wildlife studies of the bio-toxic influence of xenoestrogens with wildlife-specific estrogen receptors.

Keywords: Estrogen receptor; Ligand efficiency; Ligand potency; Transcriptional activation; Xenoestrogen

1. Introduction

Xenoestrogens, one type of endocrine disrupting chemical (EDC), can mimic the action of physiological estrogens through interaction with estrogen receptors, possibly resulting in health problems in humans and wildlife (Daston et al., 1997). Xenoestrogens thus have generated sufficient public concern to be included in the Food Quality Protection Act (Public Law 104–170) by the Environment Protection Agency. This trend has stimulated the development of various assays for the assessment of xenoestrogens, which are widely present in pesticides, plasticizers, synthetic hormones, and food supplements. Many assays have

Abbreviations: EDCs, endocrine disrupting chemicals; *vb*ERα, estrogen receptor alpha of *Varicorhinus barbatulus*; *vb*ERβ2, estrogen receptor beta 2 of *Varicorhinus barbatulus*; PCR, polymerase chain reaction; 4-NP, 4-nonylphenol; 4-*t*-OP, 4-*t*-octylphenol; BPA, bisphenol A; BBP, benzyl butyl phthalate; di-*n*-BP, di-*n*-butyl phthalate; DDT, 1,1,1-Trichloro-2,2-bis(*p*-chlorophenyl)ethane; DDE, 1,1-dichloro-2,2-bis(*p*-chlorophenyl) ethylene; PCBs, polychlorinated biphenyls; E1, estrone; E2, 17β-estradiol; ERE, estrogen response element; YES, yeast estrogen screen; SD, synthetic dropout; 5′-RACE, 5′-rapid amplification of cDNA ends; 3′-RACE, 3′-rapid amplification of cDNA ends; NCBI, the National Center for Biotechnology Information; DMSO, dimethyl sulfoxide; ONPG, *o*-nitrophenyl-D-galactopyranoside; OD, optical density; *vb*ERα-YES, *vb*ERα transformed yeast estrogen screen; *vb*ERβ2-YES, *vb*ERβ2 transformed yeast estrogen screen.

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been developed for a primary screening of EDCs. Significant progress in human estrogen receptor-mediated assays has allowed identification of the mechanism and genome-wide signatures of xenoestrogen-specific target genes (Terasaka et al., 2004; Moggs, 2005). Therefore, the desperate need to assess xenoestrogen impact on wildlife can be met by taking advantage of the well-established, nearly standardized human ER-mediated assays for a rapid, low-cost and wildlife-specific assessment strategy.

Estrogen exerts its function through interaction with membrane-associated receptors or intracellular ERs (Hall et al., 2001). A general mechanism of ER activation involves diffusion of estrogen into the cell nucleus, binding of estrogen to the ER, and dimerization of ERs to form a transcription factor activating transcription of estrogen response element (ERE)-containing target genes to control many cellular responses. Estrogen action is generally mediated by two ER subtypes, alpha (ERα) and beta (ERβ) (Dechering et al., 2000). Although ERα and ERβ share more than 60% sequence homology and cooperate at the molecular level in estrogen-responsive pathways, they differ in ligand affinity and transcriptional activity (Kuiper et al., 1997). For example, the physiological estrogen binds with higher affinity to murine ERα than to murine ERβ (Tremblay et al., 1997). Moreover, different functional effects are observed in rat uterine studies showing that ERβ can antagonize the proliferative functions of ERα (Weihua et al., 2000). Binding of E2 to the ERα receptor activates gene transcription, while binding of E2 to the ERβ receptor shows an inhibitory effect on transcription through the promoter regulatory AP1 site. Binding of the ER antagonist tamoxifen to ERβ at the AP1 site activates, while binding to ERα inhibits (Paech et al., 1997). In addition, the two subtypes differ in expression patterns and transcriptional actions on certain response elements, leading to species-, cell-, and promoter-specific actions of estrogens and antiestrogens (Kuiper et al., 1998). Significant differences in response occur through the two estrogen pathways depending on environmental chemicals. Two types of ERβ, namely β1 and β2, have been identified in teleost (Hawkins et al., 2000; Ma et al., 2000; Menuet et al., 2002), but the physiological roles of these ERβ subtypes are largely unknown. Therefore, the necessity of delineating the differential effects of xenoestrogens on different ER subtypes in wildlife, and the assessment of both subtypes as EDCs, could provide full-scope understanding of the potential risk.

Different in vitro assays, some even applied to samples from the fields, were developed to pre-screen for EDCs (Rehmann et al., 1999; Fang et al., 2000; Garcia-Reyero et al., 2001) based on the idea that the ER signaling pathway is evolutionarily conserved from yeast to human (Metzger et al., 1995). Recombinant yeast cells were designed to contain the exogenous ER receptor of interest as well as the responsive reporter stably integrated into the yeast genome. The measurement of reporter activity is a function of how xenoestrogens activate the ER, which in turn transcribes the ERE controlled reporter that brings about a color reaction (Balmelli-Gallacchi et al., 1999), bioluminescent activity (Leskinen et al., 2005) or green fluorescent protein activity (Bovee et al., 2004a). The human ER-mediated yeast estrogen screen (YES) is a reliable and robust assessment method and BLYES (bacterial luminescence yeast estrogen screen) is a modified method to shorten the reporter detection time (Sanseverino et al., 2005). Since first developed by Routledge and Sumpter (1996), the YES assay has been widely used for in vitro assays to detect xenoestrogenic compounds. This paper aims to modify this well-known tool by switching the central concerns from human to wildlife via the usage of fish ERs in this assay. Moreover, most of these methods were focused only on ERα, which may underestimate the ligand influence on other ER subtypes as was found for phytoestrogens (Harris et al., 2002).

2. Materials and methods

2.1. Fish treatment

The Taiwan cyprinid fishes, *Varicorhinus barbatulus (V. barbatulus)*, were purchased wild-caught from the fishery in Nanjuang (Miaoli, Taiwan). Adult female fishes (about 7–8 inches of body length) in a semi-recirculating tank at room temperature were fed a commercial fish diet and treated with 40 μg/l 17β-estradiol for two weeks before sacrifice to induce over-expression of ER genes. Fish livers were removed and were immediately frozen in liquid nitrogen, and total RNA was extracted using Trizol reagent (Gibco-BRL, Gaithersburg, MD) following the manufacturer's instructions.

2.2. Cloning the full length ER genes

To isolate the estrogen receptor cDNA of *V. barbatulus*, we utilized a strategy that was based upon the common identities of ER homologs from six different fish species including gilthead seabream (Accession No. AJ006039), medaka (Accession No. D28954), rainbow trout (Accession No. AJ242740), channel catfish (Accession No. AF061275), Atlantic croaker (Accession No. AF298181), and goldfish (Accession No. AF061269). ClustalW software (Thompson et al., 1994) was used for multiple sequence alignment of these gene sequences to identify conserved nucleotide sequences. Two degenerate primers (primer 1 and 2; see Table 1) based on the region overlapping the DNA and hormone binding domains were designed. Total RNA from fish liver was reverse-transcribed using the Superscript II (Invitrogen) one-step reverse transcriptase PCR kit and random primers. Polymerase chain reaction (PCR) amplification was carried out with the initial denaturation at 94 °C for 3 min, and each sample was subjected to 30 cycles of denaturation for 30 s at 94 °C, annealing at 55 °C for 30 s, and extension for 1 min at 72 °C. The generated PCR fragment was purified and cloned into the