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USE OF *XENOPUS LAEVIS* AS A MODEL FOR INVESTIGATING IN VITRO AND IN VIVO ENDOCRINE DISRUPTION IN AMPHIBIANS

YUE-WERN HUANG,* JASON B. MATTHEWS, KIRSTEN C. FERTUCK, and TIM R. ZACHAREWSKI
Department of Biochemistry and Molecular Biology and National Food Safety and Toxicology Center, Michigan State University, East Lansing, Michigan 48824, USA

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Abstract—The estrogenic activity of 17β-estradiol (E_2), α-zearalenol (α-ZEA), genistein (GEN), and 4-*t*-octylphenol (4-*t*-OP) was investigated using *Xenopus laevis*–based assays. All test compounds competed with [^{3}H]E_2 for binding to a recombinant *Xenopus* estrogen receptor (xER) with the following relative affinities: E_2 > α-ZEA > 4-*t*-OP > GEN. The ability of these compounds to induce xER-mediated reporter gene expression was then assessed in MCF-7 human breast cancer cells cotransfected with a Gal4-xERdef chimeric estrogen receptor and a Gal4-regulated luciferase reporter gene. Luciferase activity was increased 30- to 50-fold by 10 nM E_2 relative to that in solvent control. Maximal reporter gene activity induced by 10 nM α-ZEA was 54% of that induced by E_2; however, the activity did not increase following doses of up to 10 μM. A dose of 1 μM 4-*t*-OP induced 23% of the maximal reporter gene activity induced by E_2, whereas 10 μM GEN induced activity to the same level as E_2. A dose-dependent increase in vitellogenin (VTG) mRNA expression was observed in *Xenopus* treated intraperitoneally with E_2 at 0.05 to 5 mg/kg/d for three consecutive days, with the maximal induction observed in the group receiving 1 mg/kg/d. The α-ZEA, GEN, and 4-*t*-OP also significantly induced VTG mRNA expression, although at higher doses. These results demonstrate the utility of *X. laevis* as an amphibian model to assess the estrogenic activity of endocrine disruptors.

Keywords—Xenopus Estrogen receptor Competitive binding Gene expression Vitellogenin

INTRODUCTION

Many chemicals have been identified in the environment that mimic the activities of endogenous estrogens, such as 17β-estradiol (E_2). These chemicals, termed environmental estrogens, include herbicides and pesticides from residential and agricultural runoff [1]; effluents from sewage treatment plants, pulp and paper manufacturing, paint, and plastic products [2–4]; personal-care pharmaceutical products [5–7]; phytochemicals in plant-based food [8–10]; lampricide [11]; and pyrethroid insecticides [12,13].

Vitellogenin (VTG), an egg yolk precursor protein, normally is found only in the plasma of adult females during egg formation in oviparous species, but its synthesis can be induced in males following exposure to estrogens [14,15]. Thus, induction of VTG in the plasma of male oviparous vertebrates has served as a useful biomarker for assessing exposure to xenoestrogens in turtles [16], fish [17,18], and frogs [19,20]. In addition to VTG induction, exposure to estrogenic endocrine disruptors also has caused malformations of the head and abdomen as well as altered tissue differentiation at critical developmental stages and changed sex ratios in frogs [20–22].

Studies have shown that α-zearalenol (α-ZEA), genistein (GEN), and 4-*t*-octylphenol (4-*t*-OP) interact with estrogen receptors (ERs) and elicit estrogenic responses in oviparous animals in both in vitro and in vivo models. For example, α-ZEA, a metabolite of the mycotoxin zearalenone, induces the synthesis of VTG and estrogen-inducible eggshell proteins in primary salmon hepatocytes [23] as well as in exposed quail [24] and salmon [25]. A phytoestrogen in soy, GEN induced VTG synthesis in sturgeon [26]. In addition, 4-*t*-OP, a widely used surfactant that has been detected in river water [27], induces VTG in catfish hepatocytes and adversely affects breeding in medaka [28,29] as well as enhances feminization of sexual differentiation in frogs and fish [17,19]. In rodent models, these compounds induce markers of estrogen action, such as uterine weight, peroxidase activity, and lactoferrin gene expression [30–32]; however, data concerning their comparative activity in anurans are lacking.

In the present study, three complementary African clawed toad (*Xenopus laevis*)–based in vitro and in vivo assays were used to assess the estrogenic endocrine-disrupting activities of α-ZEA, GEN, and 4-*t*-OP. *Xenopus*, a commercially available model that is amenable to laboratory investigation, has been designated as a model aquatic anuran species by the Endocrine Disrupting Screening and Testing Assessment Committee of the U.S. Environmental Protection Agency. As well, a large volume of background information regarding this species can be found in the literature, including details concerning the regulation of vitellogenesis.

In the present study, a recombinant glutathione-*S*-transferase (GST) *Xenopus* ER (ligand-binding region) fusion protein was constructed and used to compare the relative binding affinities of E_2, α-ZEA, GEN, and 4-*t*-OP. A recombinant ER/estrogen reporter gene expression assay was then developed to assess the capability of these compounds to induce *Xenopus* ER-mediated activity. Finally, in vivo semiquantitative reverse transcription–polymerase chain reaction (RT-PCR) studies were conducted using the induction of hepatic VTG mRNA in male *Xenopus* as a biomarker of estrogenic activity. The in vivo VTG mRNA analysis combined with the in vitro receptor-binding and gene expression assays offer an effective means

* To whom correspondence may be addressed (huangy@umr.edu).
The current address of Y. Huang is Department of Biological Sciences, University of Missouri–Rolla, 105 Schrenk Hall, 1870 Miner Circle, Rolla, MO 65409-1120, USA.

of comprehensively investigating potential estrogenic endocrine disruptors in amphibians.

MATERIALS AND METHODS

Chemicals

The E_2, α-ZEA (2,4-dihydroxy-6-[6α,10-dihydroxy-trans-1-undecenyl]benzoic acid μ-lactone; purity, 97%), and dimethyl sulfoxide (DMSO) were obtained from Sigma (St. Louis, MO, USA). The 4-*t*-OP (4-(1,1,3,3-tetramethylbutyl)phenol; purity, 97%) was purchased from Aldrich Chemical (Milwaukee, WI, USA). The GEN (4′,5,7-trihydroxyisoflavone; purity, 97%) was purchased from Indofine Chemical (Somerville, NJ, USA). The [2,4,6,7,16,17-^{3}H]E_2 ([^{3}H]E_2; 123 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). Vent DNA polymerase was purchased from New England Biolabs (Beverly, MA, USA). Restriction enzymes and deoxynucleoside triphosphates (dNTPs) were obtained from Roche (Indianapolis, IN, USA). SuperScript II Reverse Transcriptase, Taq polymerase, Trizol reagent, and Dulbecco modified Eagle medium were purchased from Invitrogen (Carlsbad, CA, USA). Fetal bovine serum was obtained from Intergen (Purchase, NY, USA). All other chemicals were of the highest quality available from commercial sources.

Construction plasmids

The plasmid pGEX-xERdef (amino acids 259–587) was constructed by PCR amplification of the plasmid pCMV5XER1 (kindly provided by David Shapiro, Department of Biochemistry, University of Illinois, Urbana, IL, USA) using the primers xfa (5′-aaaactcgagccgggggcattcgaaaggatcgca-3′) and xra (5′-aaaaggtaccgagctctcatactgtgctttgtaagctcact-3′). The fragment was digested with *Eco*RI and *Xho*I and ligated into the similarly digested GST expression vector, pGEX6p3 (Amersham Biosciences, Piscataway, NJ, USA). The plasmid pGal4-xERdef (amino acids 248–587) was constructed by PCR amplification as described above using the primers xfb (5′-aaaactcgagccgggggcattcgaaaggatcgca-3′) and xrb (5′-aaaaggtaccgagctctcatactgtgctttgtaagctcact-3′). The fragment was digested with *Xho*I and *Sal*I and ligated into the similarly digested eukaryotic expression vector containing the DNA-binding domain of the yeast transcription factor Gal4, pG4MpolyII (kindly provided by P. Chambon, Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP/Collège de France, BP 163, 67404 Illkirch Cedex, CU de Strasbourg, France.) The PCR amplification was performed essentially as described previously [33] using Vent DNA polymerase in a reaction mixture containing Thermopol buffer, 200 μM dNTPs, 1 mM $MgSO_4$, 500 nM primer, and 1.25 U of polymerase, which was heated to 94°C for 5 min, followed by 35 cycles of 94°C for 45 s, 60°C for 45 s, and 72°C for 105 s The sequence of each construct was confirmed by restriction enzyme digest and ABI/Prism automated sequencing (Perkin-Elmer Applied Biosystems, Foster City, CA, USA).

Expression and purification of GST-xER fusion protein

Overnight cultures of *Escherichia coli* strain BL21 (Amersham Biosciences) containing the GST-xERdef construct were diluted 1:100 in 500 ml of Luria Bertoni broth (1% [w/v] tryptone, 0.5% [w/v] yeast extract, and 1% [w/v] NaCl, pH 7.5 overall) containing 100 mg/ml of ampicillin and incubated at 37°C with constant shaking. The cells were grown to an optical density of approximately 1.0 at 600 nm and induced with isopropylthio-β-galactoside at a final concentration of 1 mM. The induced cultures were incubated for 4 h at 37°C, then pelleted by centrifugation at 1,000 *g* for 10 min at 4°C. Cell pellets were resuspended in 25 ml of buffer A (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 3 mM ethylenediaminetetracetic acid, 5 mM 1,4-dithiothreitol, 50 mM NaCl, and 10% [v/v] glycerol, pH 7.5 overall) containing 0.1 mg/ml of lysozyme, 100 mg/ml of phenylmethylsulfonyl fluoride, 10 mg/ml of leupeptin, and 10 mg/ml of pepstatin A. Cells were then lysed by three sonications on ice for 15 s each time, separated by 10-s intervals. Polyoxyethylene 20-sorbitan (Tween20®; Fisher Scientific, Hanover Park, IL, USA) was added, to a final concentration of 0.1%, to the cellular debris and incubated for 30 min at 4°C under constant shaking. Cell debris was pelleted by centrifugation at 20,000 *g* for 40 min at 4°C. Supernatants were stored at −80°C until further use. The supernatants containing the GST fusion proteins were applied to an XK16 (Amersham Biosciences) column containing glutathione (GSH) sepharose pre-equilibrated with buffer A at a constant flow rate of 0.5 ml/min at 4°C. After adsorption of the protein, the GSH sepharose was washed with 100 ml of buffer B (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 3 mM ethylenediaminetetracetic acid, 5 mM 1,4-dithiothreitol, 150 mM NaCl, and 10% [v/v] glycerol, pH 7.5 overall). Bound proteins were eluted in 25 ml of buffer C (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 3 mM ethylenediaminetetracetic acid, 5 mM 1,4-dithiothreitol, 150 mM NaCl, and 10% [v/v] glycerol, pH 8.0 overall) containing 10 mM GSH. The partially purified protein was concentrated to a 1-ml final volume using Millipore® Ultrafree-15 filter columns with a 50-kDa molecular weight cutoff (Millipore, Bedford, MA, USA). Protein concentration was determined using the Bradford [34] method. Protein was diluted to 0.5 mg/ml and stored at −80°C until further use. Partially purified fusion proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis using a 4% stacking and 10% separating gel. Proteins were visualized by Coomassie brilliant blue R250 staining.

Competitive ligand-binding assay

Receptor-binding assays were performed as described previously [35] with the following modifications: Partially purified GST-xERdef (300 ng) was diluted in TEGD buffer (10 mM Tris [pH 7.6], 1.5 mM ethylenediaminetetraacetic acid, 10% [v/v], glycerol, and 1 mM 1,4-dithiothreitol) containing 1 mg/ml of bovine serum albumin and incubated at 4°C for 2 h with 1–25 nM [^{3}H]E_2. Fusion protein preparations were diluted to ensure 10,000 dpm of total binding. Binding assays were initiated by adding 240 μl of protein preparation to glass tubes containing 5 μl of DMSO and 5 μl of [^{3}H]E_2. Bound [^{3}H]E_2 was separated from unbound [^{3}H]E_2 using a 96-well filter plate and vacuum pump harvester (Perkin-Elmer, Meriden, CT, USA). The amount of radioactivity was measured using a TopCount luminescence and scintillation counter (Perkin-Elmer).

For competitive binding assays, GST-xERdef fusion protein was incubated at 4°C for 24 h with 5 μl of 10 nM [^{3}H]E_2 and 5 μl of unlabeled competitor (final concentration, 100 pM to 10 μM). Bound [^{3}H]E_2 was separated from unbound [^{3}H]E_2 and the amount of bound radioactivity determined as described above. Each concentration of unlabeled competitor was tested in quadruplicate, and at least three independent experiments were conducted. Results are expressed as the percentage spe-

cific binding of [^{3}H]E_2 versus log-transformed competitor concentration. Analysis was performed using nonlinear regression with the single-site competitive binding option of GraphPad® Prism 3.0 (GraphPad Software, San Diego, CA, USA). Reported median inhibitory concentration (IC50) values represent the calculated concentration of test compound required to displace 50% of the [^{3}H]E_2 from the fusion protein.

Cell culture, transfection, and reporter gene assay

The MCF-7 human breast cancer cells were provided by L. Murphy (University of Manitoba, Winnipeg, MB, Canada). Cells were maintained in Dulbecco modified Eagle medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 20 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethane sulfonic acid, 100 IU/ml of penicillin, 500 μg/ml of gentamicin, 100 μg/ml of streptomycin, and 2.5 μg/ml of amphotericin B. Cells were grown at 37°C in a 4% CO_2 humidified environment.

Transfections were performed as described previously [36,37]. Briefly, MCF-7 cells were allowed to attach for 6 h in six-well culture dishes in media supplemented with 6% dextran-coated, charcoal-stripped fetal bovine serum. Cells were transiently transfected with three plasmids using the calcium phosphate coprecipitation method [38]: 1.5 μg of 17m5-G-Luc and Gal4-xER vectors (kindly provided by P. Chambon), 1.5 μg of Gal4-xERdef, and 0.1 μg of pCMV-lacZ, a β-galactosidase expression vector (Amersham Biosciences) used for normalizing transfection efficiency. After 18 h, cells were treated with test compound dissolved in DMSO, with the total DMSO concentration not exceeding 0.1%. The cells were harvested after 24 h of treatment, and luciferase and β-galactosidase activities were measured using standard protocols [38,39].

Each treatment was performed in duplicate, and two aliquots were assayed from each well. Thus, means and standard deviations were calculated using four measurements. Independent experiments were conducted at least three times, and results were expressed as the percentage of the maximal induction induced by E_2. GraphPad Prism 3.0 was used for graphical analyses, including calculation of median effective concentration (EC50) values, which denote the concentration of test compound required to induce 50% of the maximal response induced by E_2.

Animal husbandry and treatment

Adult male African clawed toads (*X. laevis*; age, two to three years) were purchased from Xenopus 1 (Dexter, MI, USA). The initial body weights ranged from 31.7 to 78 g, with an average of 48.0 ± 9.0 g (mean ± standard deviation). Five to 10 individuals were randomly assigned to treatment groups, with more animals in the high-dosage groups to compensate for predicted higher variances of mean responses. Animals in the same treatment group were housed in a 10-gallon aquarium with 8 gallons of charcoal-filtered water and were acclimatized for one week before treatment. Toads were fed chopped beef ad libitum, and water was changed daily. All procedures for animal housing, handling, and dissection adhered to the guidelines provided by the Animal Use Committee at Michigan State University.

The E_2, α-ZEA, 4-*t*-OP, and GEN were each tested once. Animals were fasted 24 h before treatment. Each toad was injected intraperitoneally with a volume of 2 ml/kg for three consecutive days (i.e., days 1, 2, and 3) containing E_2, α-ZEA, or 4-*t*-OP in corn oil or with vehicle alone. Because of insolubility in corn oil, GEN was dissolved in DMSO, and the control group in that experiment was injected with 100% DMSO. An E_2 dose of 1 mg/kg/d was used as a positive control in all experiments. Animals were anesthetized using 3-aminobenzoic acid ethyl ester (MS-222) on day 12 based on previous results [40] indicating that VTG mRNA and protein were maximally induced by E_2 at this time. On dissection, an 80- to 100-mg piece of liver was excised and stored at −80°C.

VTG mRNA measurement

The thawed liver was quickly minced and homogenized in 1 ml of Trizol using a Polytron tissue homogenizer (Kinematica, Lucerne, Switzerland) for three pulses of 15 s each at 85% output. Total RNA extraction was performed by the Trizol method according to the manufacturer's instructions.

The RT, ^{32}P-labeling of forward primers, and PCR were performed as described previously [41]. The following primers were used: VTG, 5′-aaaagagctcggttggtgaatatgtccctggag-3′ and 5′-aaaatctagaatgcagataatgacacgccattgc-3′ (amplifies a 608-bp product between nucleotides 4332 and 4939 of the cDNA); and β-actin, 5′-aaaagagctcgaatcatgtggcatccatgaaacc-3′ and 5′-aaaatctagagcattacaaaattcccaacagtgtg-3′ (amplifies a 501-bp product between nucleotides 1386 and 1886). Samples were run on a 5% polyacrylamide gel, which was dried and exposed to a Molecular Dynamics (Sunnyvale, CA, USA) storage phosphor screen for 19 h. The signals were quantified using a Molecular Dynamics Storm 820 scanner and ImageQuaNT v.4.2a software (Amersham Biosciences). The VTG mRNA levels were normalized to those of β-actin, which was assumed to be unaffected by treatment.

Statistical analyses

When applicable, the normalized VTG levels were analyzed using an analysis of variance followed by the Fischer's multiple pairwise comparison procedure of Systat v. 5.04 (Systat Software, Evanston, IL, USA). A log transformation was performed to achieve homogeneity of group variances using Bartlett's test.

RESULTS

The bacterially expressed GST-xERdef was found to migrate according to its predicted molecular weight of 64.5 kDa (Fig. 1A). It appears to show a doublet. The higher-molecular-weight band most likely represents the full-length product, whereas the lower-molecular-weight band may result from proteolytic cleavage. The doublet also occurred in the other five species using the same recloning techniques and purification [42]. The dissociation constant (K_d), which represents the strength of binding between receptors and their ligands, for the GST-xERdef fusion protein was 7.7 ± 1.4 nM (n = 5) as determined by Scatchard analysis (Fig. 1B) [43].

The ability of α-ZEA, GEN, and 4-*t*-OP to compete with [^{3}H]E_2 for binding to GST-xERdef was investigated in vitro using a semi–high throughput competitive binding assay. All three compounds fully competed with [^{3}H]E_2 for binding to GST-xERdef at the highest concentrations examined (10^{-9} to 10^{-4} M); however, the IC50 value varied among compounds (Fig. 2A and Table 1). Concentrations greater than 10 μM were not examined because of potential solubility limitations.

The α-ZEA, GEN, and 4-*t*-OP induction of ER-mediated gene expression was determined by measuring luciferase activity in MCF-7 cells cotransfected with the Gal4-xERdef and

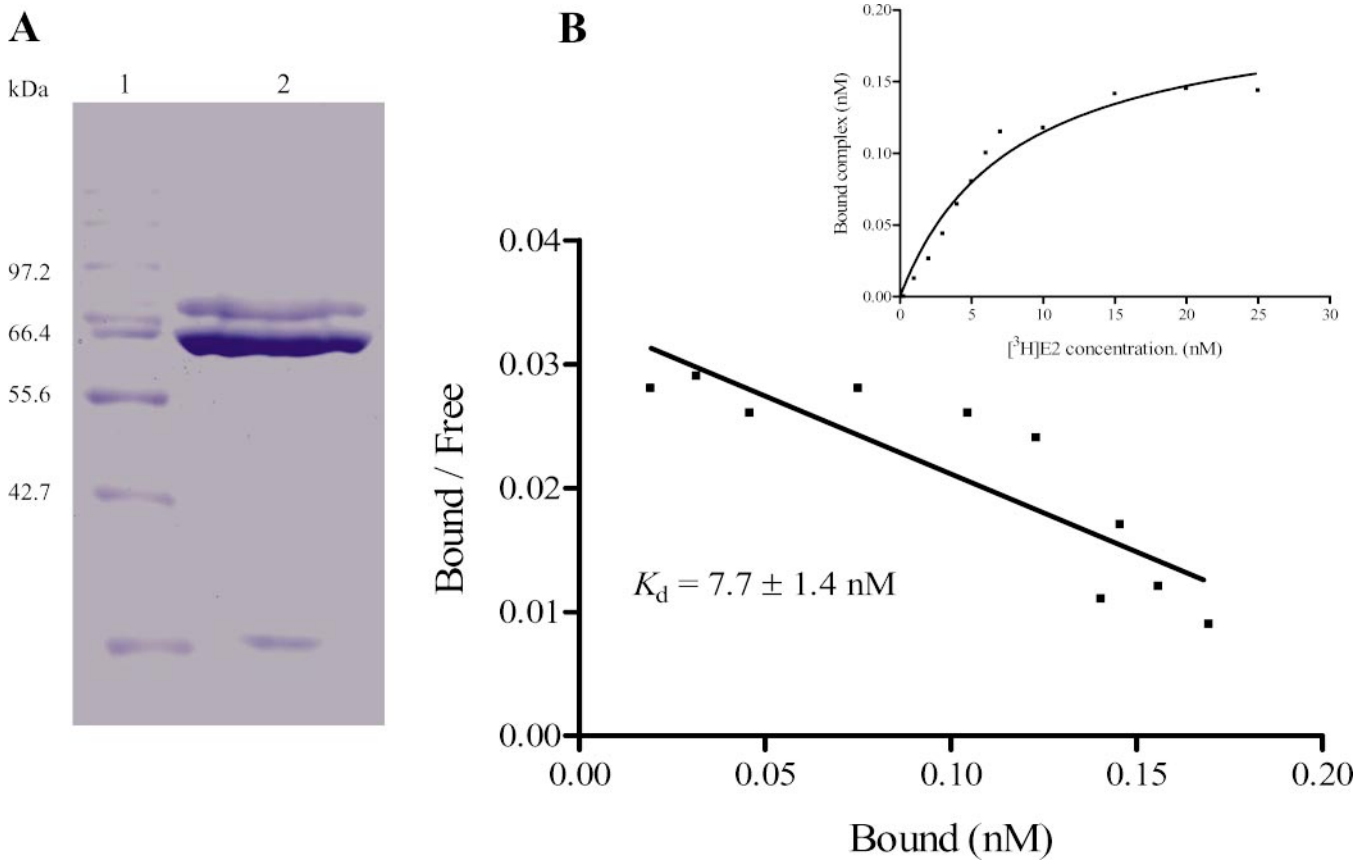

Fig. 1. (**A**) Sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis of the glutathione-*S*-transferase-xERdef (GST-xERdef) protein purified using glutathione affinity chromatography. Lane 1: standard marker; lane 2: 5 μg of partially purified GST-xERdef protein. Protein was analyzed using a 4% stacking and a 12% separating gel stained with Coomassie brilliant blue R250. (**B**) Saturation analysis of GST-xERdef fusion protein. Various concentrations of [^{3}H]estradiol were incubated with known amounts of partially purified fusion protein for 2 h at 4°C as described in *Materials and Methods*. Saturation data (inset) were plotted by the method of Scatchard. The reported K_d value was averaged from five independent experiments.

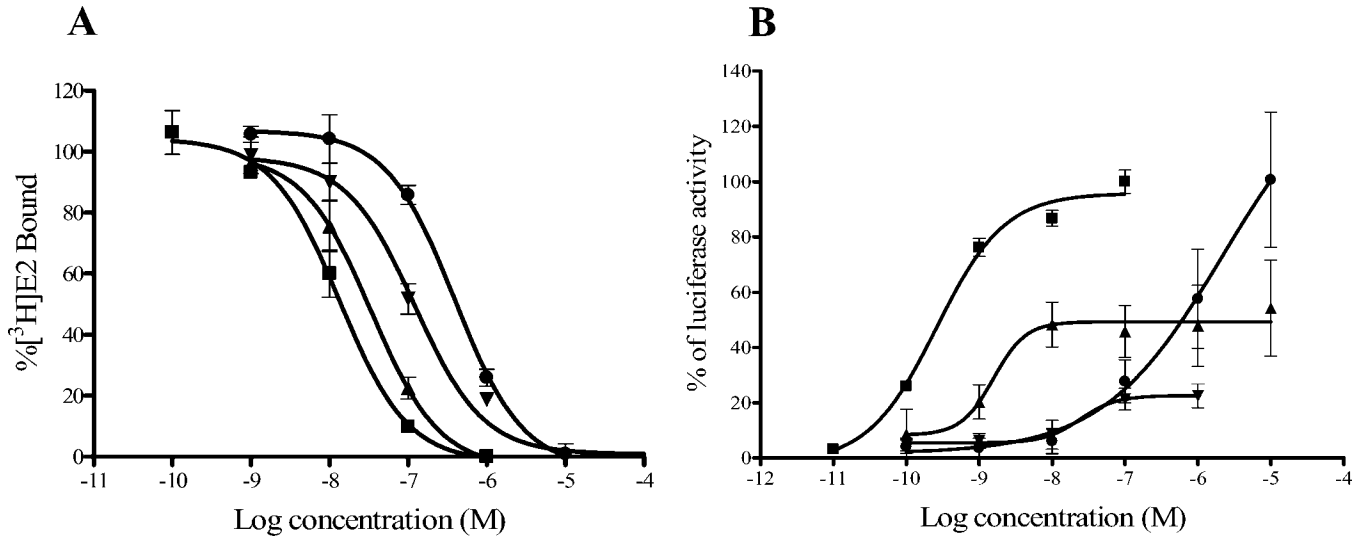

Fig. 2. (**A**) Competitive displacement curves showing competitive binding of 17β-estradiol (E_2; ■), α-zearalenol (▲), 4-*t*-octylphenol (▼), and genistein (●) to glutathione-*S*-transferase-xERdef (GST-xERdef) as described in *Materials and Methods*. The [^{3}H]E_2 is the trace of the binding assay. The results shown are from a representative experiment that was repeated at least eight times. (**B**) Induction of Gal4-xERdef-mediated reporter gene expression in MCF-7 breast cancer cells after 24 h of treatment of E_2 (■), α-zearalenol (▲), 4-*t*-octylphenol (▼), and genistein (●). Results are expressed as the percentage of maximal luciferase activity induced by E_2, normalized to solvent-treated controls. The results shown are from a representative experiment that was performed at least three times.

Table 1. Binding median inhibitory concentration (IC50) values, median effective concentration (EC50) values, percentage of 17β-estradiol (E_2) maximum induction in gene expression, and in vivo median effective dose (ED50) values[a]

	Binding assay		Gene expression		In vivo	
Chemicals	IC50 (nM)	RBA[b]	EC50 (nM)	(% E_2 max. induction)	ED50 (mg/kg)	RP[c]
E_2	13.6 ± 0.5 (27)	1	0.21 ± 0.08 (10)	100 (4)	~1.5	1
α-Zearalenol	33.5 ± 13.6 (9)	0.41	1.57 (5)	54.2 ± 17.4 (3)	>15	>0.1
4-*t*-Octylphenol	207 ± 209 (11)	0.066	24.2 (4)	22.5 ± 4.3 (4)	>300	>0.005
Genistein	424 ± 304 (9)	0.031	514 ± 274 (3)	100.7 ± 24.5 (4)	>75; <150	>0.02; <0.01

[a] The IC50 values represent the calculated concentration of test compound required to displace 50% of the [^{3}H]E_2 from the fusion protein. The EC50 values denote the concentration of test compound required to induce 50% of the maximal response induced by E_2. The ED50 values denote the dose of test compound required to induce 50% of the maximal response induced by E_2. Relative binding affinity (RBA) and relative potency (RP) of chemicals are calculated relative to those of E_2. The IC50 values, ED50 values, and percentage of induction of luciferase activity induced by maximal E_2 dose are expressed as mean ± standard deviation. The number in parentheses represents the number of replicates in the treatment group.

[b] RBA = [(IC50 for E_2)/(IC50 for chemical)]·100.

[c] RP = [(ED50 for E_2)/(ED50 for a chemical relative to E_2)]·100.

a Gal4-regulated luciferase reporter gene. The E_2 treatment of MCF-7 cells transiently transfected with Gal4-xERdef induced an increased reporter gene activity of 30- to 50-fold, with an EC50 of 0.21 ± 0.08 nM (Table 1). Although all three test compounds induced Gal4-xERdef-mediated reporter gene activity, the levels of maximum induction varied among compounds (Fig. 2B and Table 1). The GEN was able to fully induce reporter gene activity, with an EC50 of 514 ± 274 nM, whereas α-ZEA and 4-*t*-OP only induced 54 and 23%, respectively, of the maximal response achieved by E_2. Consequently, no EC50 values were determined. Although α-ZEA only induced about 54% of the maximal response of E_2, the transactivation profile revealed that α-ZEA was capable of inducing reporter gene expression at doses approximately two orders of magnitude lower than those of GEN but 10-fold higher than those of E_2. These findings were in agreement with those of the competitive binding studies, in which α-ZEA competes for binding to GST-xERdef with greater affinity than GEN. Interestingly, 4-*t*-OP, which was found to bind to the GST-xERdef with intermediate affinity, was significantly less efficacious than GEN at inducing reporter gene activity under the conditions examined.

Because in vitro studies lack the pharmacokinetic and pharmacodynamic processes that occur in vivo, the ability of α-ZEA, GEN, and 4-*t*-OP to induce VTG mRNA levels was assessed in adult male *Xenopus*. Hepatic VTG gene expression was measured 9 d following the third of three consecutive treatments with E_2, 4-*t*-OP, α-ZEA, or GEN. The levels of VTG mRNA were normalized to those of β-actin mRNA, which were assumed to be unaffected by treatment. It is important to note that any females discovered by autopsy at the end of the experiment, as well as any dead or infected individuals during the experiments, were excluded from the final statistical analysis and figures. As expected, the VTG mRNA levels increased following E_2 treatment, with a maximal increase observed at the cumulative 3 mg/kg of E_2 ($p < 0.05$, $n = 5$–6) (Fig. 3). This dose (1 mg/kg/d) was chosen as the positive control for all subsequent experiments.

In the α-ZEA experiment, induction of VTG mRNA was observed only at the cumulative dose of 15 mg/kg ($p < 0.05$, $n = 3$–9) (Fig. 4A). The normalized VTG mRNA level in the group treated with 15 mg/kg of α-ZEA was approximately one-tenth that in the groups treated with 3 mg/kg of E_2. In the groups treated with α-ZEA, the signals of VTG mRNA were at background levels in three of the six animals in group treated with 1.5 mg/kg and in 3 of the 10 animals in the group treated with 15 mg/kg. The induction of VTG mRNA was observed at or above the cumulative 75 mg/kg of GEN dosage groups ($p < 0.05$, $n = 5$–8) (Fig. 4B). The normalized VTG mRNA level in the E_2-treated group (3 mg/kg) was 4.5- and 1.4-fold higher than those in the groups treated with 75 and 150 mg/kg of GEN. However, VTG mRNA was not induced in two of the eight individuals in the 150 mg/kg dosage group. The induction of VTG mRNA was observed only in the group treated with 300 mg/kg of 4-*t*-OP ($p < 0.05$, $n = 5$–8) (Fig. 4C). The normalized VTG mRNA level in the E_2-treated group was 54-fold higher than that in the 300 mg/kg dosage group.

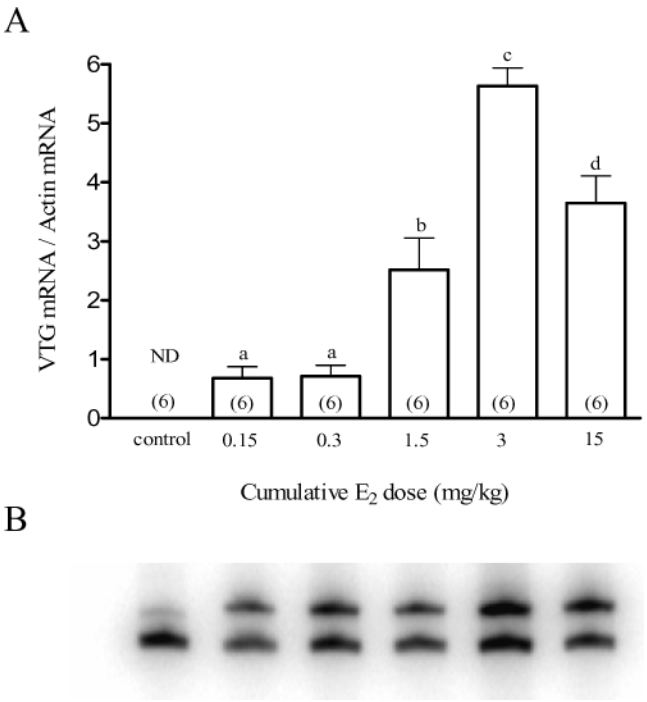

Fig. 3. Expression of hepatic vitellogenin (VTG) mRNA in *Xenopus laevis* treated with 17β-estradiol (E_2). (**A**) Intensity of the PhosphorImager signal for VTG expression normalized for β-actin expression. (**B**) Upper bands were VTG mRNA, whereas lower bands were β-actin mRNA. Within each treatment group, those values with the same letter were not significantly different from each other. Numbers of animals in each treatment group are in parentheses. ND = not detected.

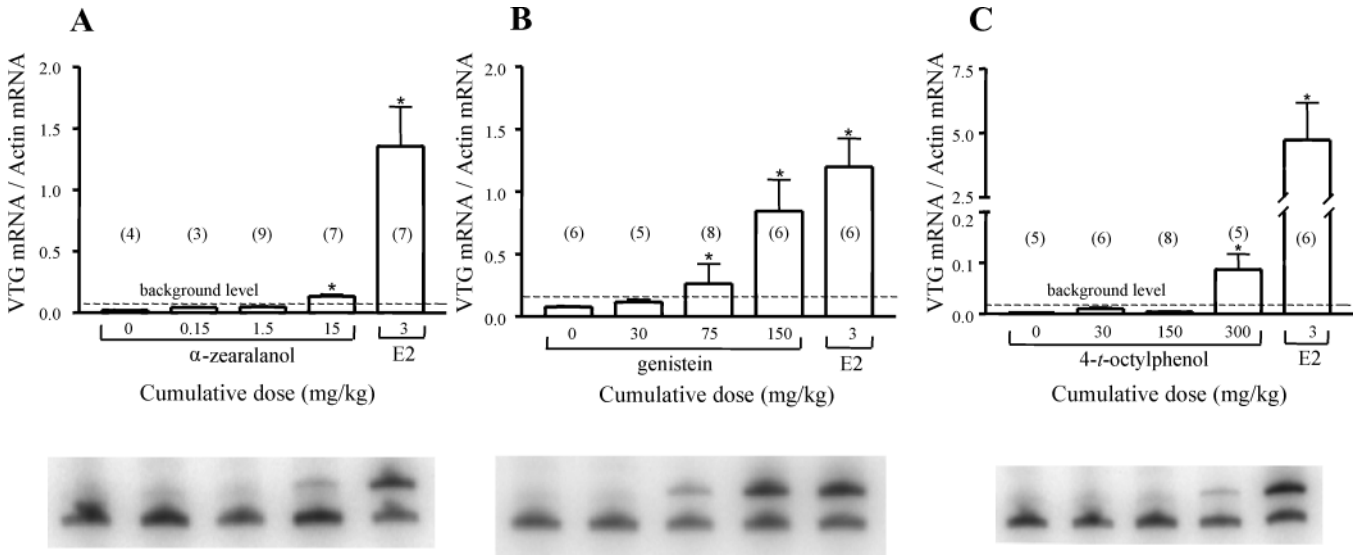

Fig. 4. (**A**) Expression of hepatic vitellogenin (VTG) mRNA in *Xenopus laevis* treated with α-zearalenol, which caused significant induction of VTG mRNA in the 15 mg/kg dose group. (**B**) Expression of hepatic VTG mRNA in *X. laevis* treated with genistein, which significantly induced VTG mRNA at and above the 75 mg/kg dose level. (**C**) Expression of hepatic VTG mRNA in *X. laevis* treated with 4-*t*-octylphenol, which caused significant induction of VTG mRNA at the 300 mg/kg dosage group. Numbers of animals in each treatment group are in parentheses. Asterisks indicate values different from those of the control ($p < 0.05$). In the lower panel, upper bands were VTG mRNA, whereas lower bands were β-actin mRNA. E_2 = 17β-estradiol.

The VTG mRNA was not induced in one individual in the 300 mg/kg dosage group. As well, one death occurred at 30 mg/kg, and two deaths occurred at 300 mg/kg. Animals at 100 mg/kg/d experienced slower-than-normal swimming behaviors and imbalanced body movement. Neither death nor abnormal behaviors were observed in any of the α-ZEA or GEN dosage groups. One should note that the normalized VTG mRNA in the experiment with 4-*t*-OP was more than threefold higher than that in experiments with α-ZEA or GEN.

DISCUSSION

The present study evaluated the estrogenic potential of α-ZEA, GEN, and 4-*t*-OP in *X. laevis* using in vitro competitive receptor-binding and induction of ER-mediated reporter gene expression assays as well as in vivo induction of liver VTG mRNA expression. The affinity of the bacterially expressed GST-xERdef fusion protein for E_2 was different from that in published reports using *Xenopus* liver cytosol. The reported K_d values of cytosolic *Xenopus* ER (xER) varied significantly, from 0.5 to 22 nM [44,45]. Differences in proteins (cytosol vs recombinant ERs), protein preparation, assay conditions, and assay methods may contribute to the variability between the present K_d values and those reported by others. Interestingly, the K_d of the GST-xERdef was more than an order of magnitude greater than that reported for full-length and truncated ERs from human α, mouse α, chicken, green anole, and rainbow trout [42], suggesting that the xER may have a lower affinity for E_2 than ERs from these species do. Additional studies need to be conducted to delineate whether the lower affinity of the xER for E_2 in *Xenopus* places them in lesser or greater danger because of the competitive nature of environmental ligands with differential binding affinities to ERs.

In competitive binding studies, α-ZEA, 4-*t*-OP, and GEN fully displaced [^{3}H]E_2 from GST-xERdef, with IC50 values that were 2.5-, 15-, and 31-fold greater, respectively, than that of E_2. In contrast to the competitive binding results, only GEN was capable of fully inducing reporter gene expression to similar levels, such as 10 μM E_2 in transiently transfected MCF-7 cells. Although the transactivation profile of α-ZEA did not exceed 60% of the maximal response elicited by E_2, a sigmoidal dose–response curve was observed, demonstrating that α-ZEA was capable of eliciting a response at lower concentrations compared with GEN; nevertheless, this response did not reach maximal induction levels achieved by 10 nM E_2. Similarly, the transactivation profile of 4-*t*-OP also exhibited a sigmoidal dose response but did not exceed 23% of the maximal response elicited by E_2. Comparable results for α-ZEA and 4-*t*-OP have been observed in reporter gene assays using Gal4-ERdef fusion proteins containing ERs from several different species [42]. The failure of α-ZEA and 4-*t*-OP to induce reporter gene activity to the same levels induced by E_2 may be the result of a failure of the compounds to reach sufficient concentration at the target site, differential interactions with or recruitment of coactivators, or using the truncated form of the xER, which lacks the NH_2-terminal and native DNA-binding domains. In the present study, MCF-7 human breast cancer cells were used, and interactions among human coactivators and transcription factors with the ligand–*Xenopus* ER complex may not be optimal, resulting in reduced transcriptional responsiveness. Future studies should consider the use of a *Xenopus* cell line, which may be more appropriate for investigating endocrine disruptor induction of gene expression in amphibians.

To account for pharmacodynamic and pharmacokinetic interactions that may affect in vivo estrogenic activity, the effects of α-ZEA, GEN, and 4-*t*-OP treatment on liver VTG mRNA levels were assessed in male *Xenopus* using semiquantitative RT-PCR. Although α-ZEA, GEN, and 4-*t*-OP significantly induced *Xenopus* VTG mRNA expression, the doses required to induce VTG mRNA and the level of induction varied significantly among the compounds. Differences observed between in vitro and in vivo studies may result from differing bioavailabilities. Furthermore, the rates and types of metabolism of these three chemicals by phase I and phase II detox-

ifying enzymes are likely to vary, thus causing unique kinetics and elimination rates in *Xenopus* and resulting in different chemical levels reaching the target site.

The α-ZEA was able to induce VTG mRNA at doses fivefold lower than those of GEN and 20-fold lower than those of 4-*t*-OP. This supports previous studies that showed the relatively potent estrogenic activity of α-ZEA in other species [25,46]. Similarly, GEN has been shown to elicit estrogenic responses in a number of in vivo models [26,30–32]. It is worth noting that animals exposed to the cumulative 300 mg/kg of 4-*t*-OP experienced slower-than-normal swimming behaviors and imbalanced body movement, suggesting potential neurotoxicity. In contrast, swimming and movement behaviors of the animals treated with α-ZEA and GEN appeared to be normal.

Some discrepancy in the normalized VTG mRNA between independent animal studies was observed. The normalized VTG mRNA in the animal experiments with α-ZEA and GEN did not differ dramatically. The normalized VTG mRNA between the 4-*t*-OP study and the E_2 dose–response study was similar but also higher than those for α-ZEA and GEN. This might result from differences in each batch of animals used and in the PCR amplification process between independent experiments. However, because we were interested in the threshold induction by each chemical and were comparing fold-induction differences between the treated groups within an independent study and against a respective positive control, this variability in the E_2 controls in independent studies was not a factor in our comparison.

In summary, RT-PCR analysis of VTG mRNA levels combined with the in vitro assays described above offer a relatively simple means of comprehensively investigating the potential estrogenic activities of select compounds in amphibians. It might be possible to use the IC50 values to predict the outcome of the transactivation profile, because the ranking order of the IC50 values is in agreement with the EC50 values. However, the percentage of the maximum response relative to E_2 does not completely follow the trend of ranking, because GEN achieved a maximum induction level comparable to E_2 at a higher dosage. Transactivation profile does not predict in vivo induction of VTG because of the disagreement between 4-*t*-OP, which might result from differential pharmacodynamic and pharmacokinetic processes in cultured cells and intact toads.

The assays presented here are of interest because of reports of a worldwide decline in amphibian populations and concerns that adverse reproductive effects caused by exposure to pollutants may be a contributing factor [47]. It is anticipated that results of these in vitro and in vivo assays will contribute important information to risk assessments accounting for the susceptibility of amphibians to estrogenic endocrine disruptors.

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