Distinct expression and activity profiles of largemouth bass (*Micropterus salmoides*) estrogen receptors in response to estradiol and nonylphenol

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Abstract

The estrogen receptor (ER) signaling cascade is a vulnerable target of exposure to environmental xenoestrogens, like nonylphenol (NP), which are causally associated with impaired health status. However, the impact of xenoestrogens on the individual receptor isotypes (α, βα, and ββ) is not well understood. The goal of these studies was to determine the impact of NP on largemouth bass (*Micropterus salmoides*) ER isotype expression and activity. Here, we show that hepatic expression levels of three receptors are not equivalent in male largemouth bass exposed to NP by injection. Transcript levels of the ERα subtype were predominantly induced in concert with vitellogenin similarly to fish exposed to 17β-estradiol (E2) as measured by quantitative real-time PCR. NP also induced circulating plasma levels of estrogen, which may contribute to overall activation of the ERs. To measure the activation of each receptor isotype by E2 and NP, we employed reporter assays using an estrogen response element (ERE)–luciferase construct. Results from these studies show that ERα had the greatest activity following exposure to E2 and NP. This activity was inhibited by the antagonists ICI 182 780 and ZM 189 154. Furthermore, both ββ and βα subtypes depressed ERα activation, suggesting that the cellular composition of receptor isotypes may contribute to the overall actions of estrogen and estrogenic contaminants via the receptors. Results from these studies collectively reveal the differential response of fish ER isotypes in response to xenoestrogens.

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Introduction

One synthetic chemical found in the environment that functions as a weak xenoestrogen is 4-nonylphenol (NP). NP is a microbial breakdown product of NP polyethoxylates (NPEs) that are most commonly found in industrial non-ionic surfactants and also used in the production of plastics, textiles, paints, emulsifiers, paper products, and cosmetics (Scott & Jones 2000, Sabik et al. 2003). It is estimated that more than 500 000 tons of NPEs are used annually (Scott & Jones 2000) with >60% entering the environment via sewage outflows (White et al. 1994, Nimrod & Benson 1996, Canadian 2000).

In fish, the estrogenic effects of NP *in vivo* are well documented. These include the induction of estrogen-responsive genes in males such as vitellogenins (vtgs) and choriogenins that are involved in normal female reproduction (Allen et al. 1999, Arukwe et al. 2001, Kannan et al. 2003), impaired gonadal development (Lin & Janz 2006), altered steroidogenesis (Harris et al. 2001), and intersex (Gray et al. 1997, Metcalfe et al. 2001, Schwaiger et al. 2002, Seki et al. 2003). These conditions have been induced in fish in controlled laboratory studies and have also been seen in wild fish found in the vicinity of sewage outflows (Allen et al. 1999, Jobling et al. 2002, Legler et al. 2002, Pettersson et al. 2005) where the main contaminants include NP.

NP has also been shown to behave as a weak estrogen in a variety of standard *in vitro* tests including proliferation assays using the breast cancer MCF-7 cell line (Soto et al. 1995, Blom et al. 1998, Laws et al. 2000), gene expression (Arukwe et al. 2000a, 2001, Thorpe et al. 2001), and recombinant reporter gene assays.
characterization of bass estrogen receptors

Materials and methods

Animals and exposures

For the in vivo exposure studies, LMB was purchased from American Sport Fish Hatchery (Montgomery, AL, USA) and maintained in accordance with the NIH Guide for the Care and Use of Laboratory Animals. For all exposures, adult male LMB (>1-year-old) were housed at the University of Florida Aquatic Toxicology Facility (Gainesville, FL, USA). Fish were acclimated 1 week prior to the experiments in aerated continuous flow through 105 gallon fiberglass tanks. Temperature (21 ± 2 °C), dissolved oxygen (6.5–8.0 mg/l), pH (7.2 ± 0.2), and total ammonia content (non-detectable) were monitored daily. All LMB were fed chow daily.

Each fish was weighed and treated with a single i.p. injection of 0.5 mg/kg E2 (Sigma), 5.0, 25.0, or 50.0 mg/kg 4-NP (>85% para isomer; Fluka Chemicals, Milwaukee, WI, USA) or dimethyl sulfoxide (DMSO) vehicle control. At various times post-injection (24, 48, and 144 h), fish were euthanized by submersion in a water bath containing 50–100 p.p.m. tricaine methanesulfonate (MS-222) followed by a sharp blow to the head followed by cervical transection following the University of Florida IACUC protocols. Blood was drawn from the caudal vein and collected in heparinized tubes for plasma E2 measurements. Bile was collected directly from the gall bladder via syringe for NP determinations. The livers were excised, finely chopped, immediately flash frozen in liquid nitrogen, and stored at −80 °C until RNA was isolated.

RNA preparation and quantitative real-time PCR

Total RNA was isolated from liver tissue and reverse transcribed as previously described (Sabo-Attwood et al. 2004). Previously validated quantitative real-time PCR (QRT-PCR) assays were used to quantify mRNA levels of ERα, ERβ, and vtg1 (Sabo-Attwood et al. 2004). Standard curves were constructed for each transcript by serial dilution of the plasmids containing IC1 182 780 and ZM 189 154. Male fish are appropriately used as models of xenoestrogen exposure since endogenous levels of E2 and hence the activation of ERs and levels of vtg are minimal to undetectable (data not shown) in non-exposed fish. The in vivo experiments were designed to examine the time- and dose-dependent expression of the ERs and vtg. The in vitro experiments confirmed our hypothesis that the expression of the three LMB ERs are differentially regulated by NP in the liver, with ERα being the most sensitive as shown previously in response to E2 (Sabo-Attwood et al. 2004).
the cloned segments for each gene. All samples were normalized to 18S rRNA as previously described (Sabo-Attwood et al. 2004). The 18S ribosomal primers and a labeled probe were purchased as a kit (PE Applied Biosystems, Foster City, CA, USA). All data were log10 transformed and graphed as the log10 of the copy number of each gene per μg of total RNA.

Plasma E₂ measurements

Plasma was isolated from whole blood by centrifugation. E₂ was extracted from the plasma with ether following a previously published method (Oakes et al. 2005). Briefly, 500 μl diethyl ether were mixed with 75 μl plasma. Following vortexing, the sample was frozen in an ethanol/dry ice bath, and the ether fraction containing the organic phase was decanted into a clean, acid-washed glass tube. The process was repeated two more times and the ether fractions were pooled. Ether was allowed to evaporate and the dried residue was dissolved in 225 μl Elisa Immunoassay (EIA) buffer (1 M phosphate solution (pH 7-4), containing 1% BSA, 4 M NaCl, 10 mM EDTA, and 0-1% sodium azide). Detection of E₂ was measured using an EIA kit specific for E₂ (Cayman Chemicals, Ann Arbor, MI, USA) following the instructions supplied by the manufacturer. A standard curve was constructed with E₂ supplied by the kit, and was used to determine the concentration of plasma E₂ in each of the samples. Sample concentrations were diluted appropriately so that values could be interpolated from the standard curve and run in duplicate. All values are presented as ng/ml of plasma.

Measurements of NP in bile samples

To detect NP in bile fluids, 100 μl pooled samples per treatment group (n=5) were enzymatically hydrolyzed, extracted with dichloromethane, derivatized to the corresponding acetates, and analyzed by gas chromatography/mass spectrometry (GC/MS) at the Enviro-Test Laboratories/Xenos Division (Nepean, Ont, Canada). Each fish bile sample was fortified with 10 μg 4-nitrophenyl-β-D-glucopyranoside as an enzymatic hydrolysis surrogate and placed in 1 ml of 4 M acetate buffer (pH 5) with 10 000 units of β-glucuronidase. Samples were incubated for 24 h at 50 °C in a sand bath with occasional shaking. After cooling, samples were fortified with 2 μg 4-cumylphenol and 1-5 ml dichloromethane. The samples were vortexed and centrifuged for 5 min. The bottom phase was extracted and placed over a sodium sulfate column. The eluate from the column was collected and pooled from three repeated extractions. The extracts were concentrated and dried under a constant stream of nitrogen. To each sample was added 2,4,6-tribromophenol as an internal standard, pyridine, and acetic anhydride. Samples were incubated at 90 °C for 1 h, cooled, and concentrated. Each sample was then dissolved in 1-0 ml toluene and analyzed by GC/MS using a DB5-MS capillary column (30 m × 0.25 mm × 0.25 μm). Quantitation standards were prepared from the 4-NP injected into the fish (Fluka Chemicals, Buchs, Switzerland). Quantitation was performed by the internal standard method using extracted ion chromatograms. All values were corrected for extraction and expressed as μg NP per mg of total protein.

Construction of LMB ER expression constructs

The cDNAs for each LMB ER (previously isolated in Sabo-Attwood et al. 2004) were cloned into the pCMV4 mammalian expression vector (Stratagene, La Jolla, CA, USA). The p Cytomegalovirus (pCMV)–ER constructs, a luciferase reporter driven by a two tandem repeat of the Xenopus vtg A2 promoter ERE (AGGTCAcagT-GACCT; gift from Dr Pierre Chambon) and a renilla luciferase construct (pRL-TK, Promega) were purified using an endotoxin-free maxi-prep kit (Qiagen).

Growth and maintenance of hepatocellular carcinoma (HepG2) cells

HepG2 cells purchased from American Type Culture Collection were maintained in Earle’s minimal essential medium (EMEM) supplemented with 0·1 mM essential amino acids (Sigma-Aldrich, St Louis, MO, USA), 2·0 mM L-glutamine, 1·0 mM sodium pyruvate, 1·5 g/l sodium bicarbonate, antibiotics/antimycotic (Hyclone, Logan, UT, USA), and 10% fetal bovine serum (Fisher Scientific, Hampton, NH, USA). The cells were cultured in a humidified incubator at 37 °C in an atmosphere of 5% CO₂ and air.

Transient transfection assay in HepG2 cells

HepG2 cells were seeded at a density of 100 000 cells per well in a 24-well plate in normal medium. Twenty four hours following plating, the culture medium was aspirated, the cell monolayers were washed once with Hank’s balanced salt solution, and the medium was replaced with media containing 10% charcoal-stripped serum. The transfection mix was made up in serum-free medium. The vector transfection mix consisted of 0·5 μg ER construct and 1 μg ERE–luciferase construct per well while the Renilla construct was used at 0·2 μg per well. Fugene 6 (Roche) was the transfectant and it was used at a ratio of 3:1 (Fugene:DNA). Each transfection mix was made up to 100 μl per well and was incubated for 15 min at room temperature. One hundred microlitres of each transfection mixture were added dropwise to the wells as directed by the manufacturer. Experiments testing the
Effect of ER mixtures were prepared similarly, except the total amount of DNA in the ER tube was increased to a total of 2 μg using the empty pCMV4 expression vector to maintain a constant mass of DNA.

Eighteen hours following transfection, the medium was aspirated and replaced with charcoal-stripped medium containing the respective treatments (E₂ (10, 25, 50, 75, 100, 500, and 1000 nM), NP (0-1, 0-5, 1, 5, and 10 μM), ICI 182 780 (5 μM), ZM 189 154 (5 or 10 μM)) keeping the amount of vehicle constant at either 0-1% for single chemicals or 0-2% for multiple chemicals in the ER antagonist assays. Treatments were allowed to continue for 48 h at which time the medium was removed, and the cells rinsed once with PBS (137 mM NaCl, 2-9 mM KCl, 8-1 mM Na₂PO₄, and 1-5 mM KH₂PO₄ (pH 7-5)). The cells were then lysed at room temperature using passive lysis buffer (Promega, Fitchburg, WI, USA) for about 20 min on a shaker. Twenty microliters of lysate were then added to wells of a 96-well Lumitrac 200 plate (Greiner Bio-One, Germany) for assay of both firefly and renilla luciferase activities using the Dual Luciferase Reporter assay system (Promega). The lysates were measured on a PlateLumino luminometer (Stratec Biomedical Systems AG, Birkenfeld, Germany) with two reagent injectors.

Model construction

The amino acid sequences used to make the structural models of LMB ERzx, ERβb, and ERβa were obtained from the National Center for Biotechnology Information (NCBI) database (accession numbers AAG44622, AAO39210, and AAO39211 respectively). Models of the LMB ER ligand-binding domains (LBDs) were built with the program Swiss-Model using the Expasy web server, http://swissmodel.expasy.org (Peitsch 1996, Guex et al. 1999, Schwede et al. 2003). The template used for the construction of all three LMB ER domains was the crystal structure of hERx (PDB accession code: 1A52; Tanenbaum et al. 1998). Modeling of E₂ into the LMB ER LBDs was performed by least-squares fitting of each individual model onto the crystal structure of hERz in complex with E₂. All model visualization and manipulation were done with the interactive graphics program Coot (Emsley & Cowtan 2004). The molecular interactions of hERz and E₂ were determined with BobScript and rendered with Raster3D (Esnouf 1999).

Statistical analysis

For QRT-PCR, the 18S rRNA-normalized copy number for each gene was log₁₀ transformed. The transformed values were then compared by ANOVA using treatment, time, and the interaction effect treatment × time (where appropriate). Where the effect of treatment was found to be significant (P ≤ 0.05), differences between the treatments were determined by Duncan’s multiple range test (DMRT). When the effect of the treatment × time interaction was significant, the data were sorted by time and the treatments were compared by DMRT (α = 0.05).

Firefly luciferase values were normalized to the respective Renilla luciferase values. For single chemicals, the fold change of the normalized luciferase values was computed by dividing each reading by the average of the vehicle response. For experiments looking at ER interactions, the fold changes were computed as with the single chemical experiments but used the empty pCMV4 expression as control. In experiments using ER inhibitors, the values of the double vehicle were used as the control.

After the fold changes were computed, the data were analyzed by ANOVA using treatment, experiment, and the interaction as the main effects using the Statistical Analysis System 8.1 (SAS Institute Inc., Cary, NC, USA). When the treatment value was found to be significant (P ≤ 0.05), further testing was done by DMRT. Data presented are means ± S.E.M. and are the result of at least three independent experiments with three wells per treatment per plate.

Results

NP increases the expression of ERz and vtg1 in LMB liver tissue

Previously, we have shown that E₂ enhances the expression of ERzx and vtg1 in LMB liver tissue in a dose-dependent manner, and induced ERβa (previously called ERγ) expression only at the highest dose of E₂ tested (2-5 mg/kg). E₂ did not enhance the expression of ERβb in those studies (Sabo-Attwood et al. 2004). To determine whether NP would have a similar effect on the transcription of these genes, we treated LMB with three doses NP (5-0, 25-0, or 50-0 mg/kg) and compared the effects with 0-5 mg/kg E₂. Exposure to 25 and 50 mg/kg NP significantly increased levels of ERzx, with the highest dose of NP increasing levels comparable with 0-5 mg/kg E₂ (Fig. 1A). No significant differences were observed in the expression levels of ERβa or ERβb at any of the doses of NP tested (Fig. 1B and C).

Vtg1, a gene known to be regulated by ERs and a common biomarker of estrogenic exposure, has previously been shown to be dose-responsive to E₂ in LMB (Bowman et al. 2002, Sabo-Attwood et al. 2004). Figure 1D shows that NP also increased the expression of vtg1 in a dose–responsive manner, with the two highest doses (25 and 50 mg/kg) giving statistically significant changes when compared with control. The
highest levels of vtg1 observed overall occurred following exposure to 0.5 mg/kg E2.

**Time-course profiles of LMB ERs and vtg1 in response to E2 and NP**

Expression levels of the three ERs in the liver were measured as a function of time at 24-, 48-, and 144-h post-exposure to single injections of NP (5, 25, and 50 mg/kg) or E2 (0.5, 1.0, and 2.5 mg/kg; Fig. 2). These times were selected based on previous studies that show peak expression of ERα between 24 and 48 h, returning to basal levels by 5–7 days post-treatment (Bowman et al. 2002). We also wanted to assess whether the ERβ isoforms were responsive to E2 and NP at other time points. Figure 2 shows that ERα transcriptional levels are significantly increased at all of the time points analyzed following exposure to the highest dose of E2 (2.5 mg/kg; Fig. 2A). Although peak levels were seen at 24 h, the expression levels remained elevated at 48 and 144 h when compared with controls. Significantly increased levels were also observed for the mid (1.0 mg/kg) and low (0.5 mg/kg) doses of E2 with peak levels occurring at 24-h post-exposure. Levels of ERα were also significantly induced by NP, but only at the two highest doses tested (Fig. 2B). In addition, the levels of ERβ were sustained by NP since peak levels were observed at 24 and 48 h compared with the pattern elicited by E2.

ERβ was upregulated by E2 only at the highest dose (2.5 mg/kg) and only at 24-h post-exposure (Fig. 2C). The other concentrations of E2 and all concentrations of NP failed to significantly induce the expression of this receptor (Fig. 2C and D). ERβ was unaltered at all times and doses of both E2 and NP (Fig. 2E and F). While the overall pattern of induction for ERα was similar in NP and E2 exposed fish, the magnitude of induction with NP was comparatively less. The data reveal that a dose of NP that is 100 times higher than E2 elicits a similar magnitude of induction of ERα transcriptional levels.
Figure 3 shows that NP increased the expression of vtg1 mRNA by 24 h and then further increased it at 48-h post-injection, indicating that the effect of NP on this gene was sustained similarly to the effects on ER$\alpha$. With 0.5 mg/kg E2 on the other hand, vtg1 expression peaked within the first 24 h and then significantly declined by 48 h, although it remained elevated when compared with control values. At 48 h, the levels of vtg1 in response to E2 were comparable with the expression elicited by 50 mg/kg NP at 24 h. As seen for ER$\alpha$, the peak levels of vtg1 obtained following exposure to E2 and NP were similar, indicating that a dose of NP 100 times greater than E2 is required to elicit similar transcriptional levels of this gene. By 144 h, the expression of vtg1 returned to control levels for both chemicals.

**Figure 2** Time course of ER isotype expression following exposure to E2 and NP. Livers from male LMB (n=5 per group) injected with vehicle (DMSO), 0.5 mg/kg E2 (circles), 1.0 mg/kg E2 (triangles), 2.5 mg/kg E2 (squares) or 5 mg/kg NP (circles), 25 mg/kg NP (triangles), and 50-0 mg/kg NP (squares) by injection (i.p.) were assayed at 24, 48, and 144 h following treatment for the expression of ER$\alpha$ (A and B), ER$\beta$ (B and C), and ER$\beta$ (E and F) by QRT-PCR. All data are presented as the log$_{10}$ transformed mean number of copies per $\mu$g of total RNA. Statistically significant differences between control and treated fish ($P\leq0.05$) for each time point are represented with an asterisk.

**Determination of NP metabolites in LMB bile**

We measured concentrations of NP metabolites in pooled samples of bile from fish exposed to 50 mg/kg NP and the results are presented in Table 1. The levels
were similar at 24 (1083.6 mg NP/mg total bile protein) and 48 h (995.5 mg NP/mg total bile protein), suggesting slow clearance of NP via biliary excretion.

**Plasma levels of E2 are increased by NP**

Although it has been suggested that xenoestrogens bind directly to ERs to activate gene transcription, it has also been shown that they can alter the synthesis or biotransformation of endogenous hormones by alternative pathways. Figure 4 shows that the highest dose of NP used in these experiments significantly increased E2 concentrations in the blood to ~1.0 ng/ml by 24 h. This was the dose and time where peak expression of ERz and vtg1 was observed. As expected, there was a significant dose-dependent increase in plasma E2 levels when fish were injected with E2 (0.5, 1.0, and 2.5 mg/kg).

**Activation of LMB ERz, ERβa, and ERβb in HepG2 cells by E2**

To assess the activity of the ERs in response to varying concentrations of E2, each ER in combination with an ERE–luciferase reporter plasmid was transfected into human HepG2 cells. This cell line contains undetectable levels of endogenous hERs (data not shown) so it is an appropriate cell line in which to assess the activity of LMB ERs. Following exposure to E2 (10–1000 nM) or ethanol as a control (0.1%), each of the three ERs was responsive in a dose-dependent manner as measured by activation of transcription of the luciferase reporter gene (Fig. 5A). ERz was the most sensitive receptor becoming distinct from vehicle at 25 nM, and it went on to reach the greatest magnitude over the ethanol control. Each of the ERβs required 50 nM E2 for receptor activity, with ERβb reaching a greater level of transcriptional activation than ERβa. The EC50 value obtained for ERz was 106.8 nM with a 95% confidence interval (CI) of 76.66–148.9 nM. For the ERβs, the EC50 values were similar with ERβa at 125.7 nM and ERβba at 134.8 nM. ERβa and ERβba had 95% CIs at the EC50 of 67.82–232.9 and 88.24–205.8 nM respectively.

**Activation of LMB ERz, ERβa, and ERβb in HepG2 cells by NP**

In order to determine the effect of NP on ER activity, the LMB ERs were transfected into HepG2 cells as described previously and the cells were treated with 0.1–10 μM NP or ethanol as a control (0.1%). Figure 5B shows the results of these experiments. ERz and ERβb were most sensitive to NP. ERz responded at 0·1 μM and continued to rise in activity through 5 μM where it reached a plateau of about 9·5-fold activity over vehicle. ERβb also responded at 0·1 μM and reached a plateau at 1·0 μM resulting in 7·5-fold activity over vehicle. ERβa did not respond as the dose–response curve was not
different to that of the empty pCMV expression vector (data not shown).

ER\textsubscript{a} was found to have an EC\textsubscript{50} of 1.05 μM with a 95% CI of 0.69–1.62 μM. No EC\textsubscript{50} value could be calculated for ER\textsubscript{b} due to both the lack of significant effect of treatment and the near linear horizontal slope of the dose–response curve. ER\textsubscript{b} had an EC\textsubscript{50} of 0.27 μM with a range of 0.17–0.43 μM. Based on EC\textsubscript{50} values, NP was found to be about 10% as potent an agonist for ER\textsubscript{a} as E\textsubscript{2}, while it was found to be about 50% as potent as E\textsubscript{2} for ER\textsubscript{b} (Fig. 5C).

**Effects of antagonists ICI 182 780 or ZM 189 154 on ER activation by E\textsubscript{2}**

To further characterize the LMB ERs, transfected HepG2 cells were treated with 500 nM E\textsubscript{2} with or without the ER antagonists ICI 182 780 (ICI; 5 μM) or ZM 189 154 (ZM; 5 or 10 μM) based on concentrations used in other fish models (Hornung et al. 2003), or with ethanol as vehicle (0.1%). Figure 6 shows that ICI at 5 μM (tenfold molar excess over E\textsubscript{2}) was able to completely inhibit ER\textsubscript{a} activity to the level of vehicle alone. ICI was unable to significantly

![Graphs showing ER activation](image)

**Figure 5** Activation of LMB ERs in response to E\textsubscript{2} and NP. Eighteen hours following transfection, cells were treated with (A) E\textsubscript{2} (10, 25, 50, 75, 100, 500, or 1000 nM) or (B) NP (0.1, 0.5, 1, 5, or 10 μM) in a vehicle of ethanol (0.1%) in charcoal-stripped serum containing medium. Cells were lysed 48 h following treatment with 100 μl passive lysis buffer and 20 μl cellular lysate were assayed for firefly and renilla luciferase activities. The ratio of firefly luciferase to renilla luciferase was computed for each sample, and the fold change of each chemical concentration was calculated compared with vehicle. The data were analyzed by ANOVA and differences between concentrations were determined by Duncan’s multiple range test (α = 0.05). Data presented are the means ± s.e.m. from triplicate wells from three independent experiments. Means with different letters were found to be significantly different. (C) Calculated EC\textsubscript{50} from the curves presented above.
decrease the activity of ERβa, while it was able to decrease ERβb activity by ~55% versus E2 alone. ZM at either concentration (10- or 20-fold molar excess over E2) was able to completely inhibit the transcriptional activation for all three ERs stimulated by E2. On its own, ZM was unable to stimulate transactivation of any of the ERs, showing that it acts as a pure antagonist for this response.

Modulation of ERα activity by ERβα or ERββ

In certain tissue types, both ERα and ERβ are found co-expressed and it has been hypothesized that their differential response to estrogentic ligands in control of downstream transcriptional activity is regulated, in part, by the formation of ER heterodimers. To address this possibility, a set of experiments were performed to measure the effect of increasing the amount of either ERβα or ERββ against a constant amount of ERα. Figure 7 shows that in both sets of experiments, ERα alone stimulated a 16-fold increase in transcriptional activity whereas ERβα and ERββ alone were each able to stimulate a four- and eight-fold increase in activity respectively. The addition of 0.1 µg (20% of ERα) ERβα resulted in ~37-5% decrease in activity, while the addition of 0.1 µg ERββ resulted in ~25% decrease in activity. Adding 0.25 µg (50% of ERα) ERβα resulted in a 44-4% decrease of overall activity, while the addition of
0.25 μg ERβb resulted in no greater loss in activity. Co-transfection of an equal amount of either ERβ or ERα resulted in no further significant decrease in activity.

Model of ERα with E2

Structural models of proteins may help us explain differences in ER activity in response to estrogens. The percentage sequence identity among the LMB ERα, ERβ, and ERβa LBDs compared with that of the hERα is 46, 45, and 44% respectively. A least-squares fit of the three LMB ER LBD models resulted in an average root-mean-square deviation, for all atoms, of <1·0Å. Using the hERα as a model receptor, we show which amino acids make contact with E2 (Fig. 8). This model predicts residues from hERα that make hydrogen bonding interactions (yellow ball-and-stick) and form a hydrophobic-binding pocket for E2 (red half-moons). Respective residues that contact E2 in the LMB ERs are identical to hERα except the bass ERα contains a methionine (M) instead of a leucine (L) at position 349.

Discussion

We have previously shown that injection of LMB with increasing concentrations of E2 resulted in the induction of ERα, ERβa (to a smaller extent), and vtg1 in liver tissue (Sabo-Attwood et al. 2004), indicating that the impact of E2 on the transcriptional regulation and activation of the different ER isotypes are not identical. The goal of this study was to observe changes in the expression and activity of three LMB ER isotypes and vtg1 following exposure to the environmental contaminant NP. Based on previous studies, we hypothesized that NP would act as a weak estrogen and induce transcription of E2-activated genes (ERα and vtg1) through an ER-mediated process. The production of vtg in male fish has been clearly linked to reproductive impacts in multiple species and populations (Miller et al. 2007). Altered reproductive biomarkers of LMB from contaminated field sites have been documented (Sepulveda et al. 2002, 2003, Schmitt et al. 2005) and causally linked to xenoestrogen exposures (at least in part).

Regulation of LMB ER isotype and vtg1 expression by NP in vivo

Results from our studies reveal that vtg1 and only ERα mRNA levels were significantly increased following exposure to NP (25 and 50 mg/kg), whereas no change in the levels of ERβa and ERβb was observed for any of the doses at the time points measured. This is consistent with our previous report showing a strong correlation between the induction of vtg1 and ERα mRNA during the reproductive cycle of female LMB and in males injected with E2 (Sabo-Attwood et al. 2004). Interestingly, in our previous study, ERβa levels were significantly increased only by our highest dose, 2·5 mg/kg E2 (Sabo-Attwood et al. 2004). None of the NP doses tested stimulated ERβa levels in the present study, indicating that even the highest dose of NP was much less potent than 2·5 mg/kg E2. Similar levels of ERα and vtg1 induction were noted in response to 0·5 mg/kg E2 and 50 mg/kg NP, indicating that NP is ~100-fold less potent by comparison. Based on this observation, our highest dose of NP would unlikely produce a similar
response as 2.5 mg/kg E$_2$. In a recent study (Filby & Tyler 2005), aqueous exposures of fathead minnows to E$_2$ failed to induce transcription of ER$\beta_a$ in the liver, suggesting that this isotype is not strongly regulated at the transcriptional level by E$_2$ at the concentrations naturally found in fish throughout the reproductive cycle. It is noteworthy to mention that we have documented high levels of ER$\beta_a$ (and ER$\beta_b$) in LMB ovarian tissue when plasma levels of E$_2$ were low, indicating that the transcriptional regulation of the ER$\beta$s may not be steroidal in nature and is cell-type specific (Sabo-Attwood et al. 2004). In contrast, goldfish ER$\beta_a$ mRNA was inducible by NP in the liver (Soverchia et al. 2005) following chronic water exposure. The reason for this discrepancy is unclear but may be related to route and duration of exposure in the two experiments, or alternatively using primers that did not distinguish the different receptor isotypes. The expression of ER$\beta$s mRNA was not measured in those previous reports, which seems to be the most highly expressed receptor in response to both E$_2$ and NP in bass and other fish (Filby & Tyler 2005).

Overall, the similar expression profiles for the three ERs and vtg1 in LMB liver tissue exposed to NP compared with E$_2$, albeit at lower magnitudes, confirms NP is weakly estrogenic. Furthermore, these data suggest that ER$\beta$s is the only isotype strongly auto-regulated by estrogens at the transcriptional level in the liver, an observation that may be due to varied promoter-response elements and cell-specific pools of co-effector proteins. Although the transcriptional response of the ERs and vtg1 was similar between E$_2$ and NP, the time-course plots suggest that activation of ER$\beta$s and vtg1 by NP is more sustained. Differences in distribution and clearance of E$_2$ and NP may contribute to this observation. Increased levels of NP metabolites were detected in LMB bile, consistent with other observations that show biliary excretion is a major route of NP removal from fish (Coldham et al. 1998, Arukwe et al. 2000b). A chief clearance pathway of E$_2$ and NP includes the generation of glucuronide conjugates and it is possible that exposure to NP, especially at high doses, saturates this pathway, resulting in prolonged circulation of E$_2$. This may contribute to the increased levels of plasma E$_2$ observed in NP-treated LMB. This level of induction (1 ng/ml) is similar to physiological levels seen in female LMB during active reproduction and corresponds to peak levels observed in this species during the annual reproductive cycle. Although NP has been shown to interact directly with the ERs of numerous species (White et al. 1994, Kuiper et al. 1998, Balaguer et al. 1999, Nishikawa et al. 1999, Gale et al. 2004), a few reports have suggested that the estrogenic effects of NP in vivo are the result from altered levels of endogenous steroids (Acevedo et al. 2005, Matsumura et al. 2005, Soverchia et al. 2005). Exposure of fathead minnows to NP also resulted in increased levels of plasma E$_2$ with a concomitant decrease in testosterone (Soverchia et al. 2005). Others have shown that NP enhanced 20$\alpha$- and 20$\beta$-hydroxy-steroid dehydrogenase activities and inhibited glucuronidation pathways involved in the clearance of E$_2$ in carp microsomes (Thibaut & Porte 2004), and

![Figure 8 Predicted model of LMB ER$\beta$ with ligand. (A) Ribbon diagram of LMB ER$\beta$ with E$_2$ bound. Backbone with predicted secondary structure is shown in grey with estradiol in yellow ball-and-stick. Figure was generated with PyMOL. (B) Molecular interactions of hER$\alpha$ and E$_2$. E$_2$ and residues from hER$\alpha$ that make hydrogen bonding interactions are shown in yellow ball-and-stick and are as labeled. Residues that form a hydrophobic-binding pocket are indicated as red half-moons and are also as labeled. The model was generated with BobScript and rendered with Raster3D. For readers of the printed journal a colour version of this figure can be found at http://dx.doi.org/10.1677/JME-07-0038.](image-url)
increased the expression of aromatase (Cytochrome P450-19; CYP19) in fish (Min et al. 2003, Kazeto et al. 2004) but not in mammalian models (Odum et al. 2001). These observations suggest that NP-induced alterations in steroid synthesis, metabolism, and clearance, in addition to direct activation of ERz contribute to the estrogenic effects of this compound in fish. The role of these individual components in NP-induced endocrine alterations remains to be determined.

**Differential activation of LMB ER subtypes by E2 and NP**

To understand potential functional differences of the ER isotypes, a reporter gene assay was developed to assess their activity in liver cells. Using this system, we observed little difference for the computed EC$_{50}$ values among the bass ERs (106–8–134·8 nM). This is similar to work with zfERs by Bardet et al. (2002). These values are much higher than those reported for the hERs where EC$_{50}$ values for the hERz and hERb range from 0·1 to 4 nM and from 5 to 10 nM respectively, depending on the reporter assay employed (Hall & McDonnell 1999, Gaido et al. 2000). In general, fish have higher circulating concentrations of E$_2$ (600–3900 pg/ml for females (Orlando et al. 1999, Gross et al. 2002, Sabo-Attwood et al. 2004)) in comparison with human females which range from 23 to 361 pg/ml depending on time of the menstrual cycle (during pregnancy, normal concentration can be as high as 35 000 pg/ml; Simpson & MacDonald 1981). Previous studies have shown that the rainbow trout ER binds E$_2$ with tenfold less affinity than hER, which may be a result of varied protein structure, and may explain why higher levels are noted in fish. Furthermore, it has been suggested that since the body temperature of fish is generally less than 37 °C in the HepG2 assays may cause them to need far more ligand than what may usually be required by warm-blooded species (Matthews et al. 2001).

Although the EC$_{50}$ values were similar, the level of activity varied for each ER. ERz was most responsive to E$_2$, followed by ERb; whereas ERb was minimally affected. At equivalent concentrations, ERb activity was approximately half that observed for ERz. This is consistent with the differences in activity for the hERs reported previously in HepG2 cells, where the activity of hERb was 20–60% of hERz (Hall & McDonnell 1999). Since ERz and ERb of other species, including mammals and fish, bind E$_2$ with similar affinity, and amino acids that directly interact with E$_2$ are almost identical (Fig. 8), their differential activity is more likely due to distinct interactions with endogenous co-activator and co-repressor proteins.

The effects of the model endocrine disruptor, NP, on transcriptional activation of the bass ERs showed that ERz and ERb respond to NP in a dose-dependent manner, however, ERb did not. This finding is different from data published by Legler et al. (2002) who found that all three of the zf ERs, including ERb (ERz in their report) were responsive. The pattern of ER inducibility was also different between bass and zf. Bass ERz and bass ERb had EC$_{50}$ values of 1·052 and 0·27 respectively, compared to values of 1·48 and 0·42 respectively for the zf homologs. Legler et al. (2002) also compared the zf ER response with NP with hERs finding EC$_{50}$ values of 0·11 and 0·145 nM for ERz and ERb respectively. One reason for the differences in these two reports may be the use of different cell types. Legler et al. used HEK293 cells, while HepG2 cells were used in the present study. Also using HepG2 cells, Yoon et al. (2000) showed that NP can stimulate activity of hERz via either AF-1 or AF-2 domains using receptor deletion mutants, finding that the AF-1 domain can be activated by lower concentrations of NP than the AF-2 domain. However, the wild-type full-length ERz responded more like the AF-2 domain. Taking these findings into account, the differences in the amino acid composition of the receptor isotypes in the AF domains may be responsible for the lack of response through ERb.

**Inhibition of ER activity by the antagonists ICI 182 780 and ZM 189 154**

ERz was inhibited in activity by the ER antagonist ICI 182 780 when the cells were treated with tenfold molar excess over E$_2$. When the same treatment was done with the ERb, we did not see a significant decrease in ERb activity and only a 55% decrease in receptor activity for ERb, where the activity still remained significantly higher than vehicle. When the receptors were challenged with ZM 189 154, we found the receptors fully inhibited with a tenfold molar excess of inhibitor to ligand. In addition, the ZM compound did not exhibit any agonist activity in these assays. The differences observed in the abilities of the two 'pure' estrogen antagonists to inhibit transactivation of the bass ERs was not anticipated, since amino acid residues known to be involved directly in E$_2$ binding in the LBDs are identical in the three receptors (Fig. 8). There must be other amino acid residues which directly interact with the antagonists and recruit variable pools of co-repressor proteins that contribute to these differences.

**Modulation of ERz activity by ERb and ERz**

Tremblay et al. (1999) first demonstrated that hERz/β heterodimers can occur following treatment in gel shift...
assays from transfected cells. They then showed that when transfected in combination, a 33% decrease in transcriptional activity through a basal ERE promoter occurred. Hall & McDonnell (1999) went on to show that by increasing the amount of hERβ, the overall activity of hERα can be decreased in direct relation to the concentration of hERβ, and that this only occurs at sub-saturating concentrations of E2. This is similar to our findings where the addition of increasing concentrations of either of the ERβs with ERα reduced the amount of transcriptional activity observed with ERα alone. These observations imply that the ratio of ERs in a given cell plays a role in target gene activation. It has been suggested that the AF-1 domain of hERβ has a repressor function that is the cause of the overall decrease in activity of the ERα/ERβ heterodimeric complex (Hall & McDonnell 1999). Further studies are needed to address the molecular basis of these observations, but these results stress the importance of cellular composition for ER transcriptional regulation of downstream target genes. We are currently in the process of validating antibodies designed to specifically distinguish the three ERs. These will be useful for many applications in vivo including tissue and subcellular localization of the ERs, dimerization complexes, and assessment of activation by gel shift assays.

Overall conclusions
Based on the data presented, there is evidence showing that the two ERβ subtypes in bass are non-redundant, having differing responses to different ligands, and that these in turn are different from ERα. Although the mechanistic in vitro data compliment the in vivo observations observed in this study, we realize the complexity of the mechanism in vivo and are aware of two assumptions, which require further testing in vivo. These assumptions are that an increase in mRNA levels of the ERs translates to protein and that the ER isoforms are present in the same cells thereby modulating each others activity in response to ligands. If these assumptions are correct, by altering the amount(s) of receptor present, as well as their activity by agonists and antagonists in the environment, homeostasis in the fish can be seriously compromised, affecting not only the reproductive health, but also the visceral health of this species. The finding that NP did not stimulate ERβa in the assays in this report, indicates that the deleterious effects occurring through ERα-mediated pathways does not occur through direct activation of ERβa in the liver. Even more broadly, the combinatorial complexity possible with having three distinct ERs in nearly every organ system in the body does suggest that further study of other endocrine active compounds is necessary as proposed by the EPA in the Endocrine Disruptor Screening and Testing Advisory Committee.

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