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Transcriptional activity and biological effects of mammalian estrogen receptor ligands on three hepatic estrogen receptors in Mozambique tilapia

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ABSTRACT

Like other fish species, Mozambique tilapia has three forms of estrogen receptor, ERα, ERβ1, and ERβ2. A primary function of 17β-estradiol (E_2) in oviparous species is the hepatic induction of the yolk precursor protein, vitellogenin (Vg). To characterize the roles of ERs in Vg production, transactivation assays and an *in vivo* study were carried out utilizing agonists for mammalian ERα and ERβ, and an antagonist for mammalian ERα, propyl-pyrazole-triol (PPT), diarylpropionitrile (DPN), and methyl-piperidino-pyrazole (MPP), respectively. ERα was more sensitive and responsive to PPT than ERβ1 or ERβ2 in transactivation assays. All ER isoforms indicated equivalent responsiveness to DPN compared with E_2, although sensitivity to DPN was lower. MPP exhibited antagonistic action on transactivation of all ER isoforms and reduced the E_2 effect on Vg and ERα 48 h post-injection. DPN increased ERα and Vg expression and plasma Vg post-injection, whereas PPT was without effect; DPN seems to stimulate Vg production through activation of ERα. The ligand binding domain of all tilapia ER forms shares only 60–65% amino acid identity with human ERα and ERβ. This, together with our results, clearly indicates that agonistic or antagonistic characteristics of PPT, DPN and MPP cannot be extrapolated from mammalian to piscine ERs.

1. Introduction

Estrogens are involved in a host of physiological processes including reproduction, growth and development [1,2]. In most vertebrates, ligand bound estrogen receptors (ER), ERα and ERβ, act as transcription factors that positively or negatively regulate DNA synthesis in the presence of co-factors [3]. First identified in Atlantic croaker, a third form of nuclear ER is being characterized in a growing number of fish species [4]. This ER is a result of a duplication of the ERβ gene; previously termed ERα, ERβ, and ERγ, fish ERs were renamed ERα, ERβ1, and ERβ2, respectively, to conform to zebrafish nomenclature [5,6]. Many tissues, of both male and female fish, co-express ER subtypes and levels can vary among tissues, genders, ontogeny, and reproductive and behavioral states [5,7–9].

Vitellogenin (Vg), a female-specific hepatic yolk precursor protein, is a commonly studied endpoint for oviparous organisms exposed to estrogens or estrogenic compounds. Vg is not detectable in male plasma under most circumstances, however, after exposure to estrogens, phytoestrogens or estrogenic compounds, plasma Vg increases and remains elevated [10]. Like ER, three forms of Vg have been identified in several fish species [11,12]. It remains unclear which ER forms mediate Vg production and whether there is a relationship among multiple ERs and multiple Vgs [13]. Several studies provide support for ERα-mediated vitellogenesis in fish [5,14–17], while others suggest ERβ carries out that role [18,19].

The development of compounds with specific binding capabilities for ERα and ERβ has provided valuable tools for elucidating the roles of ER subtypes in mammals, particularly in the brain and vascular system [20,21]. Propyl-pyrazole-triol (PPT) is a mammalian ER agonist with a 410-fold greater affinity for ERα over ERβ; PPT activates transcription solely through mammalian ERα [22]. Diarylpropionitrile (DPN), on the other hand, has a 70-fold higher binding affinity for ERβ compared with ERα; DPN has a 170-fold greater ability to initiate transcription through ERβ in mammals [23]. Methyl-piperidinopyrazole (MPP) is a pyrazole compound that strongly antagonizes ERα, with a 200-fold greater binding affinity for ERα than for ERβ; MPP shows no activation of either receptor subtype [24].

Recently, Leaños-Castañeda and Van Der Kraak [18] described the *in vitro* effects of PPT, DPN and MPP on Vg production by rainbow trout hepatocytes. The test compounds all achieved complete displacement of specific [^{3}H]-estradiol binding from the nuclear

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extract of ERα and ERβ1 from the liver. Nevertheless, only E_2 and DPN induced Vg synthesis, and the authors suggested that Vg production is mediated through ERβ. The aims of this study were to characterize effects of mammalian ER agonists, PPT and DPN, and the ERα antagonist MPP, on the transcriptional activation of three ERs in Mozambique tilapia, and to describe their effects on gene expression of three ERs and Vgs and on plasma Vg levels after intraperitoneal injection.

2. Methods

2.1. Rearing

Juvenile tilapia (*Oreochromis mossambicus*) used for the *in vivo* study were obtained from brood stock at the Hawaii Institute of Marine Biology, University of Hawaii. They were maintained in 700 L freshwater flow-through tanks at 26 °C ± 2 °C under natural photoperiod and were fed approximately 2% of the body weight per day with Silver Cup Trout Chow (Nelson and Sons, Murray, UT). Experiments were conducted in accordance with the principles and procedures approved by the Institutional Animal Care and Use Committee, University of Hawaii.

2.2. Molecular cloning of estrogen receptors

For Mozambique tilapia ERs, two conserved amino acid regions in the DNA-binding domain (GYHGVW) and the ligand binding domain (NKGM/IEHL) of vertebrate ERs were selected and degenerate oligonucleotides were used as primers for PCR. As a template for PCR, the first strand cDNA was synthesized from 2 μg total RNA isolated from female Mozambique tilapia liver. After amplification, an additional primer set corresponding to two amino acid sequences, CEGCKAF and NKGM/IEHL, was used for the second PCR.

The amplified DNA fragments were subcloned with TA-cloning plasmid pCR2.1 vector (Invitrogen, Carsbad, CA), sequenced using a BigDye terminator cycle sequencing kit (PE Applied Biosystems, Foster City, CA) and analyzed on the ABI PRISM 377 automatic sequencer (PE Applied Biosystems). The 5′- and 3′-end of ERs were amplified by rapid amplification of the cDNA end (RACE) using a SMART RACE cDNA amplification kit (BD Biosciences Clontech, Palo Alto, CA). A single full-length transcript of the open reading frame was then amplified using the primer set at the 5′-untranslated region and 3′-untranslated region.

2.3. Construction of plasmid vectors

The full-coding regions of Mozambique tilapia ERs obtained from this study were amplified by PCR with KOD DNA polymerase (Toyobo Biochemicals, Osaka, Japan). The PCR product was gel-purified and subcloned into pcDNA3.1 vector (Invitrogen). An estrogen-regulated reporter vector containing four estrogen response elements (4xERE), named pGL3-4xERE was constructed as described previously [25].

2.4. Transactivation assay

HEK293 cells were seeded in 24-well plates at 5×10^4 cells per well in phenol red-free Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 10% charcoal/dextran-treated fetal bovine serum (Hyclone, South Logan, UT). After 24 h, the cells were transfected with 400 ng of pGL3-4xERE, 100 ng of pRL-TK (Promega, Madison, WI), and 400 ng of pcDNA3.1-ERα, -ERβ1, or -ERβ2 using Fugene 6 transfection reagent (Roche Diagnostics, Basel, Switzerland) following manufacturer's protocol. After 5 h of incubation, test compounds, 10^{-15} to 10^{-5} M E_2, PPT, DPN, or 10^{-9} M E_2 plus 10^{-7} to 10^{-6} M MPP were added to the culture medium. After 43 h, the cells were collected and the luciferase activity of the cells was measured by a chemiluminescence assay with Dual-Luciferase Reporter Assay System (Promega) according to manufacturer's protocols. Luminescence was measured using a Turner Designs Luminometer TD-20/20 (Promega). Promoter activity was calculated as firefly (*Photinus pyralis*)-luciferase activity/sea pansy (*Renilla reniformis*)-luciferase activity.

2.5. In vivo effects of DPN, PPT, and MPP

Male tilapia, weighing an average of 20 g, were fasted for 2 weeks prior to the beginning of the experiment to reduce basal plasma Vg levels (see [26]). Fish were separated into 5 groups ($n = 9$) and received a single injection of canola oil (control), E_2 (5 μg/g), DPN (5 μg/g), PPT (5 μg/g) or E_2 (5 μg/g) + MPP (25 μg/g). After 48 h, all fish were anesthetized using 2-phenoxyethanol (0.2 mL/L) and blood was collected from the caudal vasculature by a needle and syringe treated with ammonium heparin (200 U/mL, Sigma). Plasma was separated by centrifugation at 10,000 × *g* for 10 min at 4 °C and stored at −20 °C for measurement of plasma Vg. A sample of liver was frozen in liquid nitrogen and stored at −80 °C for analysis of gene expression.

2.6. Plasma Vg and gene expression of multiple ERs and Vgs

Plasma Vg levels were measured by enzyme-linked immunosorbant assay (ELISA) as previously described [11]. Hepatic gene expression of Vgs A–C and ERs α and β1 were analyzed by real-time quantitative RT-PCR, as previously described [11]. ERβ2 expression was measured using SYBR green chemistry and the following primers: 5′-CCCCCGTCCCTCACACA-3′ forward and 5′-GAACTCCTTTGGGCTCATGGT-3′ reverse. Expression of all genes was normalized to the housekeeping gene, acidic ribosomal phosphoprotein, which did not vary with treatment. Data are expressed as values relative to control levels.

2.7. Statistical analyses

Statistical analyses were conducted using one-way analysis of variance (ANOVA) followed by Fisher's least significant difference test for normally distributed data, or non-parametric Kruskal–Wallis followed by Dunn's multiple comparison test for non-normally distributed data. All analyses were conducted using the computer program STATISTICA (StatSoft, Tulsa, OK). Significance level was set at $P < 0.05$, and data are expressed as means ± SEM.

3. Results

3.1. Cloning of ERs

Full-length sequences of 3 types of ERs were cloned from Mozambique tilapia. The sequences of the three ER subtypes were identical with those recently reported by Esterhuyse et al. [27]. Comparison of ER ligand binding domain sequences shows that human and tilapia ERα share 66% identity while human ERβ shares 62% and 65% identity with tilapia ERβ1 and ERβ2, respectively (Table 1).

3.2. Transactivation assay

To examine the effect of E_2 and ER-specific agonists on Mozambique tilapia ERs, we utilized the reporter gene assay using transient transfection. E_2 added to cells transfected with ERα, ERβ1, or ERβ2

Table 1
Identity percentages between ligand binding domains (LBD) of human ERα (P03372), ERβ (NP_001428.1), and Mozambique tilapia (mt) ERα (AM284390), ERβ1 (AM284391), and ERβ2 (EU140820).

LBD	hERα	hERβ	mtERα	mtERβ1	mtERβ2
hERα	100				
hERβ	61	100			
mtERα	66	60	100		
mtERβ1	62	62	51	100	
mtERβ2	61	65	45	58	100

BLAST alignment was utilized to calculate identity values.

clones induced luciferase activity in a dose-related manner, with EC50s of 1.74×10^{-10}, 5.08×10^{-11}, and 4.23×10^{-11} M, respectively (Fig. 1). PPT and DPN, the agonists for mammalian ERα and ERβ, respectively, also induced transcriptional activity of all three genes in a dose-related manner, but with lower effectiveness. For ERα, both PPT and DPN acted at similar doses with EC50s of 1.55×10^{-8} and 1.08×10^{-8} M, respectively (Fig. 1A). In contrast, ERβ1 was more sensitive to DPN compared with PPT. The relative potency of DPN was 9.5-fold higher as compared with PPN (Fig. 1B). Further, differences in magnitude of activation were seen in DPN- and PPT-induced ERβ2 transactivities. 10^{-5} M DPN induced a 3.2-fold greater increase in ERβ2 activation compared with PPT at the same concentration (Fig. 1C).

MPP, a selective anti-estrogen for mammalian ERα, was used for examining E_2-induced Mozambique tilapia ER activity. E_2 (10^{-9} M) and MPP (10^{-7} or 10^{-6} M) were added to culture medium, and then luciferase activity from ERE-driven reporter genes was estimated. ERα and ERβ1 were more sensitive to MPP than ERβ2 (Fig. 2). 10^{-6} M MPP inhibited the E_2-induced transactivational activity of both ERα and ERβ1 significantly ($P < 0.001$). MPP had anti-estrogenic activity against ERβ2, but ERβ2 was more resistant to MPP than ERα and ERβ1. The E_2-induced transcriptional activity of ERβ2 was reduced by up to one-third by 10^{-6} M MPP ($P < 0.01$) (Fig. 2C).

3.3. *In vivo injection study*

To expand further on the effects of the mammalian ER agonists, PPT and DPN, and antagonist, MPP, in tilapia, an *in vivo* experiment was conducted. A single injection of 100 μg E_2 (5 μg/g) significantly ($P < 0.001$) increased plasma Vg from 0.7 ± 0.2 to 11.8 ± 1.3 mg/mL after 48 h, an effect that was significantly ($P < 0.05$) attenuated by the concurrent addition of MPP (25 μg/g) (Fig. 3). Although of less magnitude than E_2, DPN also significantly ($P < 0.001$) increased plasma Vg levels to 3.1 ± 0.36 mg/mL, while PPT had no effect.

Liver expression of ERα was significantly ($P < 0.001$) increased 5.6-fold by E_2 (5 μg/g); this effect was significantly ($P < 0.01$) attenuated by 25 μg/g MPP (Fig. 4A). DPN also increased ERα gene expression slightly but significantly ($P < 0.05$) by 1.6-fold. ERβ1 was significantly ($P < 0.01$) increased by DPN as well as E_2 + MPP, but not by E_2 alone (Fig. 4B). ERβ2 was not affected by any treatment (Fig. 4C). All three Vg genes showed similar changes in gene expression 48 h after a single injection of E_2, DPN, PPT, or E_2 + MPP (Fig. 5). Vgs A–C were all significantly ($P < 0.001$) increased by E_2, an effect that was reduced by MPP by 97.4%, 81.5%, and 74.3%, respectively. DPN also significantly ($P < 0.001$) increased gene expression of Vgs A-C, while PPT had no effect.

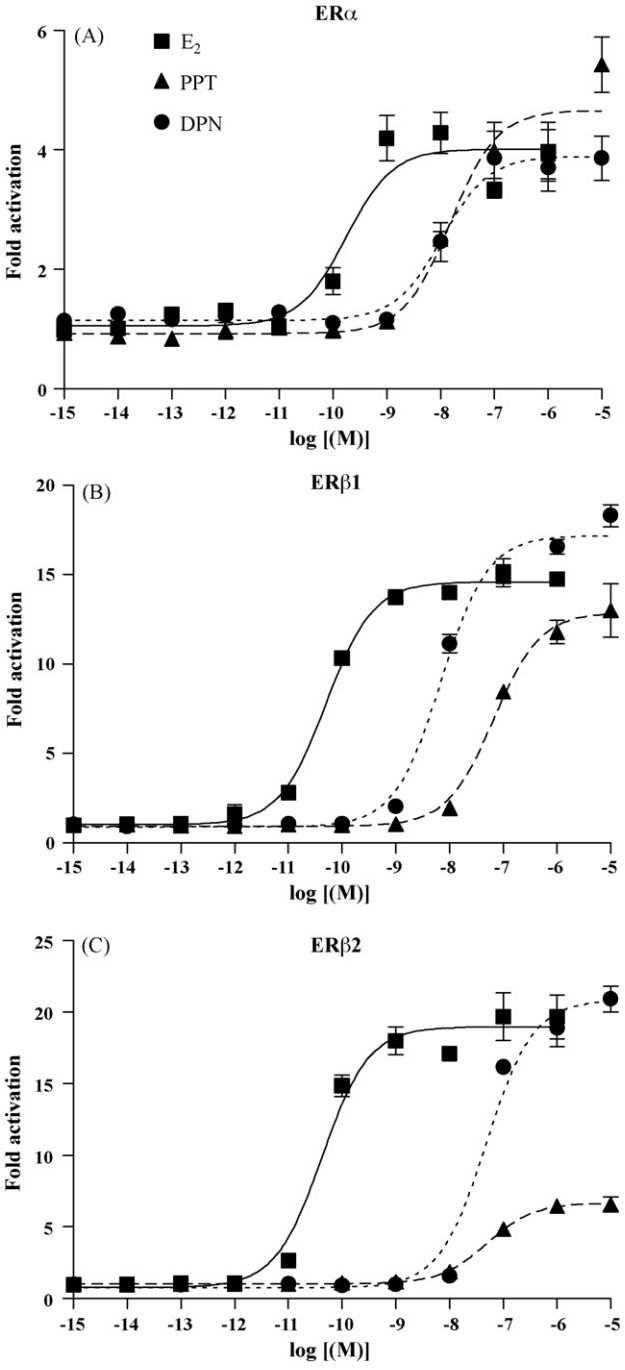

Fig. 1. Transcriptional activation of tilapia ERα, ERβ1, and ERβ2 by E_2, PPT and DPN. Changes in activation of ERα (A), ERβ1 (B) and ERβ2 (C) by 10^{-15} to 10^{-5} M E_2, PPT or DPN, the synthetic ligands for mammalian ERα and ERβ, respectively, were measured by reporter gene assays. Each point represents the mean ± SEM of triplicate assays.

4. Discussion

The importance of E_2 in vertebrate physiology is unquestionable. Nevertheless, the discovery of multiple forms of its receptor makes understanding the variety and mechanism(s) of its actions more challenging. The number of fish species discovered to have three nuclear forms of ER continues to increase. While the regulation of these genes is important and becoming more thoroughly characterized, the transcriptional activation of the genes is far less well described. Using PPT and DPN, well-characterized agonists for mammalian ERα and ERβ, we have shown that all three forms of tilapia ER bind and are transcriptionally activated by these compounds, but in a different manner than has been described for mammals. *In vivo*, DPN, an ERβ agonist in mammals, stimulated hepatic expression of ERα and Vg A–C genes as well as plasma Vg levels, in a similar fashion to E_2. PPT, the mammalian ERα agonist,

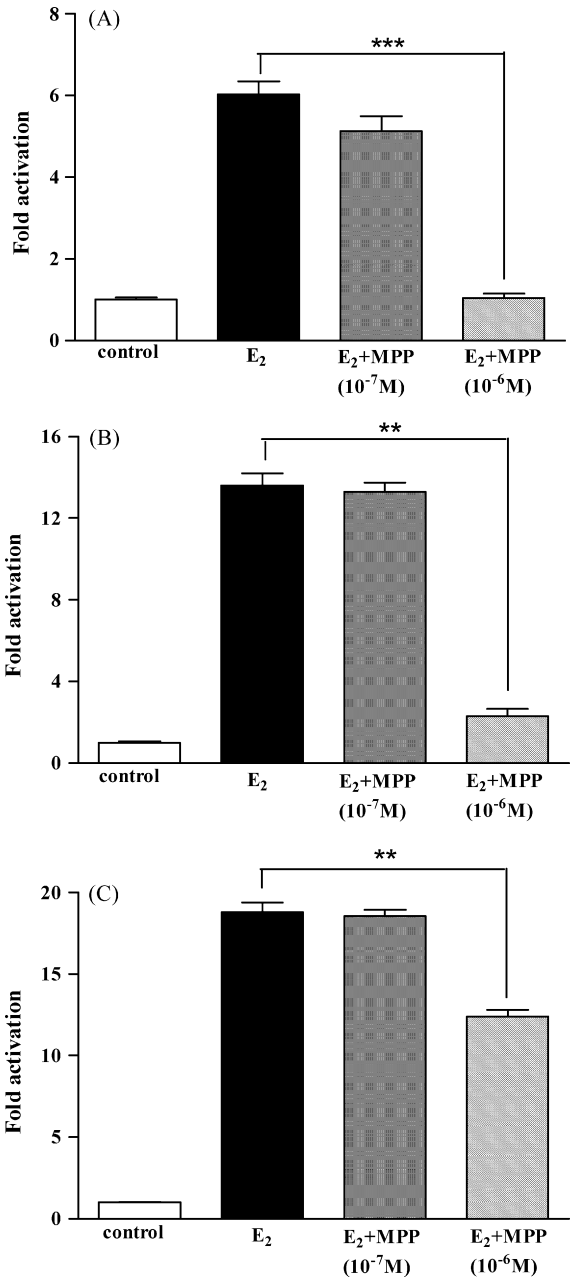

Fig. 2. Effect of MPP on E_2-induced activation of tilapia ERs as measured by reporter gene assays. At 10^{-6} M, MPP, a selective anti-estrogen for mammalian ERα, significantly inhibited activation of tilapia ERα (A), ERβ1 (B), and ERβ2 (C) induced by 10^{-9} M E_2. Data are expressed as values relative to control and each point represents the mean ± SEM of triplicate assays. **, *** Significantly different from E_2 alone at $P < 0.01$ and $P < 0.001$, respectively.

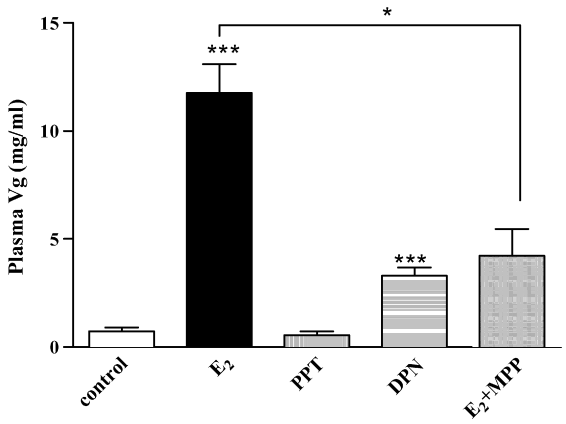

Fig. 3. Plasma Vg levels of male tilapia given a single injection of vehicle (control), 5 μg/g E_2, PPT or DPN, or 5 μg/g E_2 + 25 μg/g MPP. Fish were fasted for 2 weeks prior to the study, and plasma samples taken 48 h post-injection and estimated by ELISA. Columns represent the mean ± SEM ($n = 9$). *, *** Significantly different from control or E_2-treated fish at $P < 0.05$ and $P < 0.001$, respectively.

on the other hand, had no significant effect on hepatic expression of ER and Vg genes or on Vg production.

In female oviparous animals, an important role of E_2 is the ER-mediated stimulation of hepatic Vg production. In studies examining multiple ERs in fish, the majority suggest that ERα is the mediating form, as it is consistently upregulated by E_2 in a variety of species [5,14,15,17,28]. The results of the present *in vivo* experiment again support ERα as a mediator of Vg production with concurrent, robust increases in mRNA levels for ERα and Vgs A–C after E_2 injection. Additionally, MPP, the mammalian ERα antagonist, strongly antagonized tilapia ERα in reporter gene assays; MPP also abolished E_2 effects on ERα and Vg genes and Vg production *in vivo*. The present findings differ from those of a recent study by Leaños-Castaneda and Van Der Kraak [18], in which MPP had no effect on E_2-induced Vg protein production in male rainbow trout hepatocytes. In addition, Nelson and Habibi [13] found that MPP had no effect on E_2-induced Vg and ERα expression in goldfish hepatocytes. The discrepancy in MPP effects between the species may be due to interspecies differences in affinity for ERα or differences in experimental designs. Comparable with our study, however, DPN, induced a vitellogenic response in rainbow trout hepatocytes, while PPT showed no effect [18]. Based on the lack of PPT effect on Vg production, the authors excluded ERα as a mediator of vitellogenesis in trout. Nonetheless, results of our reporter gene assay indicate that PPT and DPN are equipotent in their activation of ERα transcription. According to Leaños-Castañeda and Van Der Kraak [18], both PPT and DPN achieved complete displacement of specific [^{3}H]-estradiol binding from the nuclear extract of ERα and ERβ1 of the rainbow trout liver. It seems highly likely that DPN may bind ERα to induce Vg production also in rainbow trout. Significant *in vivo* stimulation of expression of ERα and Vg genes as well as Vg production may indicate that DPN acts primarily as an ERα agonist in tilapia.

Human ERβ has been shown to inhibit ERα signaling in target tissues and decreases its activity [3]. Sabo-Atwood et al. [15] showed that cells transfected with largemouth bass ERα were highly responsive to E_2, increasing transcriptional activity by 16-fold. Nonetheless, with the addition of ERβ1 or ERβ2 at 20% of ERα levels, activation was reduced by 37.5% and 25%, respectively. According to Nelson and Habibi [13], ERα is induced by E_2 through activation of the ERβ subtypes in goldfish. We have previously shown that male tilapia express higher hepatic transcript levels of ERβ1 than ERα, the predominant form in females [14]. Additionally, based on C_t values from our real-time PCR assays, ERβ2 is also more highly expressed than ERα in male liver. Collectively, these results suggest that the ratio of ER subtypes is important in determining the cellular response to estrogens in fish. In our *in vivo* experiment, DPN significantly increased expression of both ERα and ERβ1. We speculate that DPN, like E_2, initially induced ERα and Vg gene expression, which was later followed by increased ERβ1. This may have resulted in a shift in the ERα/ERβ ratio and deactivation of Vg production with ERα returning to near control levels. In the same experiment, E_2 treatment caused a marked increase in ERα expression, while no significant effect was observed on ERβ1 expression. This disparity between E_2 and DPN effects may be attributed to the ability of E_2 to autoregulate and stabilize ERα mRNA, a mechanism by which E_2 can maintain elevated ERα levels and in turn, Vg production [29,30].

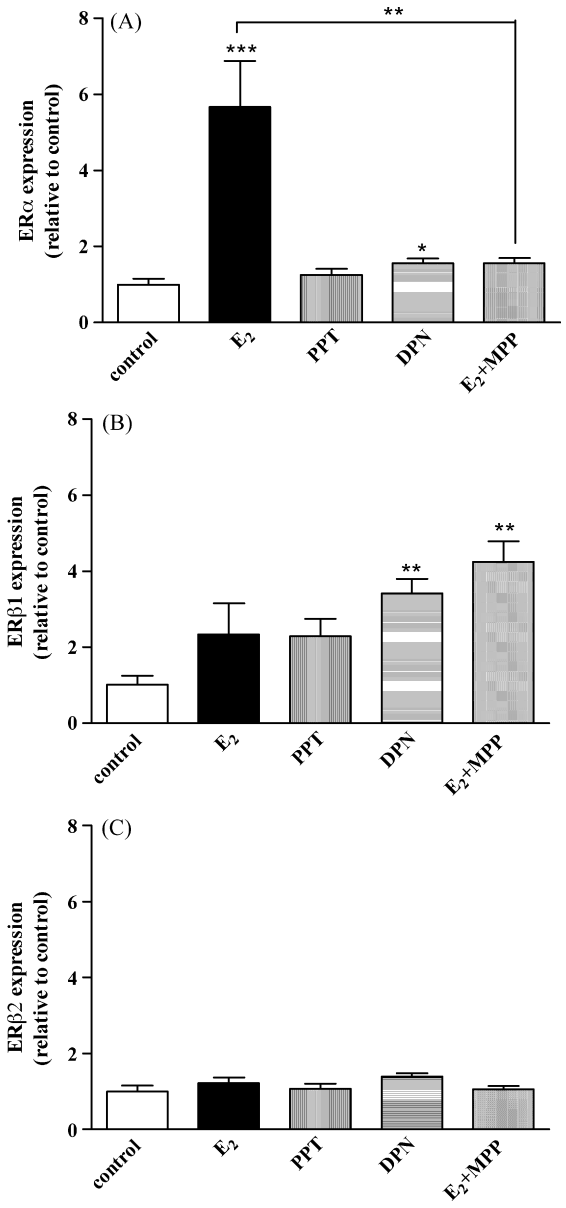

Fig. 4. Liver gene expression of ERα, ERβ1, and ERβ2 in male tilapia given a single injection of vehicle (control), 5 μg/g E_2, PPT or DPN, or 5 μg/g E_2 + 25 μg/g MPP. Livers were sampled 48 h post-injection and analyzed by real-time PCR. Data are expressed as relative values to control; columns represent mean ± SEM (n = 9). *, **, *** Significantly different from control or E_2-treated fish at P < 0.05, 0.01, and 0.001, respectively.

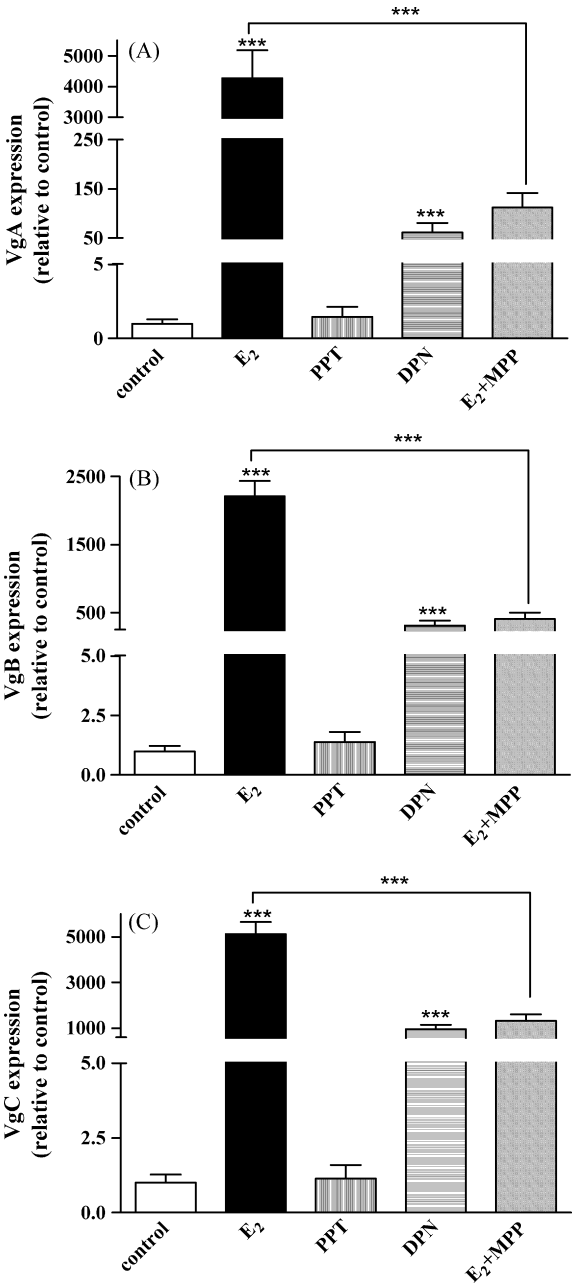

Fig. 5. Liver gene expression of Vg A, Vg B, and Vg C in male tilapia given a single injection of vehicle (control), 5 μg/g E_2, PPT or DPN, or 5 μg/g E_2 + 25 μg/g MPP. Livers were sampled 48 h post-injection and analyzed by real-time PCR. Data are expressed as relative values to control; columns represent mean ± SEM (n = 9). *** Significantly different from control or E_2-treated fish at P < 0.001.

PPT, which has a 410-fold greater affinity for mammalian ERα over that for ERβ [22], had no apparent agonistic activity in the liver of tilapia; this was unexpected, given that the transcriptional activation of ERα by PPT and DPN was approximately the same in reporter gene assays. Nevertheless, there are a host of possible explanations for the disparity between the *in vivo* and reporter gene assay results. The activity of ERs is highly dependent on the recruitment of co-activators and their interaction strength with the ligand–receptor complex [31,32]. Given that the reporter gene assay is a mammalian-based *in vitro* system, the balance of co-activators, co-repressors and cell-specific factors *in vivo* can dramatically alter ligand action. The possibility of PPT metabolism or different pharmacokinetics *in vivo* is also likely. Harris et al. [33] found that rats that received 10 mg/kg PPT had plasma levels that remained above 450 ng/mL for at least 4 h post-injection. Thus, it is probable that PPT had been cleared from circulation in our study at 48 h when samples were collected.

MPP is a potent mammalian ERα antagonist with a 200-fold greater binding selectivity for ERα over ERβ [24]. It is apparent from our reporter gene assays that MPP antagonizes all three ER forms in tilapia; 10^{-6} M MPP reduced the E_2-induced transcriptional activity by 83% for both ERα and ERβ1, while ERβ2 activation reduced by 34%. The ligand binding domain of human ERα shares 66%, 62% and 61% amino acid identity with tilapia ERα, ERβ1 and ERβ2, respectively. These homologies are likely not great enough for MPP to exhibit the high specificity it shows for mammalian ERα. Curiously, in our *in vivo* experiment, ERβ1 expression was significantly increased by the combination of 5 μg/g E_2 and 25 μg/g MPP, while E_2 alone had no effect. Nelson and Habibi [13], reported similar

results with both MPP alone, and in combination with E_2, increasing ERβ1 expression in male goldfish hepatocytes. The increased ERβ1 expression in both species may be related to differences in conformation or co-factor binding, resulting in agonist activity by MPP.

The actions of ER ligands are unique, depending on the promoter, presence of co-activators or co-repressors, cell type and species differences [3,5,34,35]. Considering the different homologies among ligand binding domains of human ERs and tilapia ERs, as shown in the current study, the differences in agonist activities in tilapia for compounds designed to bind mammalian ERs is not surprising. There was similarity, however, in antagonistic effects of MPP, although the binding to tilapia ERs appears more promiscuous than in mammals. The current results suggest that E_2 induction of multiple Vg genes is mediated primarily by ERα, functions of which may be modulated by ERβ1 and/or ERβ2. The studies described here are confined to the adult tilapia liver; E_2 and its receptors also have well-defined roles in sex differentiation and ontogeny of fishes, as well as in other tissues such as brain and gonads [36–38]. In mammals, the actions of PPT, DPN, and MPP vary, depending on the species, physiological conditions, dosing method, and tissue [39–42]. Our studies have shown that, in particular, the mammalian ERβ agonist DPN has the ability to act as a selective ER agonist, likely for ERα, in tilapia. Additionally, PPT does not appear to be a specific agonist for any one tilapia ER. Likewise, MPP has antagonistic effects on all three ER forms. These results indicate that agonistic or antagonistic characteristics of PPT, DPN and MPP cannot be extrapolated from mammalian to piscine ERs, and it is apparent that further characterization of these synthetic compounds is necessary in non-mammalian vertebrates. Nonetheless, the unique effects of PPT, DPN, and MPP on ERs and Vg production in tilapia show promise that these tools, traditionally used in mammalian research, may also help in understanding the important actions of ERs in fishes.

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