Expression Analysis of Estrogen-responsive Genes Vitellogenin 1 and 2 in Liver of Male Medaka (*Oryzias latipes*) Exposed to Selective Ligands of Estrogen Receptor Subtypes

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Vitellogenin (VTG) is a useful biomarker for detecting the estrogenic activity of chemicals in aquatic environments. However, little information is available on the regulatory mechanisms of the expression of each VTG subtype, particularly the relationship between expression patterns of VTG1/2 and estrogen receptor (ER) subtypes, such as ERα and ERβ. In this paper, we measured VTG1 and VTG2 mRNA induction in male medaka liver, which was treated with ERα-selective ligand, (17α, 20E)-3-hydroxy-17,20-[(1-methoxyethylidene)bis(oxy)]-19-norpregna-1,3,5(10),20-tetraene-21-carboxylic acid, methyl ester or ERβ-selective ligand, 2-(4-hydroxyphenyl)-5-hydroxy-1,3-benzoxazole and investigated the characteristics of ER subtype function in VTG1 and VTG2 inductions. Hepatic VTG1 mRNA was induced by ERα-selective ligands at even low concentration and maximum increases were the same as for E2. VTG2 mRNA was also increased, but its levels were very low. On the other hand, ERβ-selective ligands significantly increased VTG2 mRNA in the presence of ERα agonists. These results indicate that the expression of each VTG subtype is regulated by unique ER subtypes. VTG1 expression is only regulated by the action of ERα. In contrast, VTG2 expression is regulated by both ERα and ERβ, with ERα being essential for VTG2 gene expression and ERβ being essential for enhancement.

Key words —— vitellogenin, estrogen receptor, estrogen receptor α-selective ligand, estrogen receptor β-selective ligand, medaka

INTRODUCTION

Endocrine-disrupting chemicals and their effect on the health of wildlife and humans have recently become a major concern.[1] Hormones exert activity even at trace amount in organisms. The sex steroid hormone estrogen is involved in essential phenomena for organisms, including development, differentiation, reproduction and homeostasis, and thus estrogenic chemicals are of particular concern with regard to undesirable effects in wildlife and humans.[2, 3] Therefore, the estrogenic effects and their intensity must be evaluated in order to confirm the safety of both preexisting and novel chemicals.

For *in vivo* assay, the use of small fish has several advantages, as they can be studied throughout the entire life cycle and require short periods of time to mature. In fact, several assessment methods using medaka (*Oryzias latipes*), rainbow trout (*Oncorthynchys mykiss*), fatheadminnow (*Pimephales promelas*) and zebrafish (*Brachydanio rerio*) have been proposed by the Organization for Economic Cooperation and Development.[4, 5] When chemicals are assessed for estrogenic effects, indices include: ovary development in female fish; delay of testis development in male fish; increase in liver weight; and induction of egg yolk protein vitellogenin (VTG)

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in male fish. VTG is characteristic of mature oviparous females in the reproductive stage. In mature oviparous females, induction of vitellogenesis occurs in liver cells following to secretion of estrogen, and VTG is released into the bloodstream, where it is incorporated into endocytosis mediated by receptors. Subsequently, VTG is processed into lipovitellin, phosvitin, and β'-component through processing by proteases.[6] Immature females and males do not secrete much estrogen, and VTG induction is scarcely observed. However, VTG induction is observed in males and immature oviparous females treated with estrogen or estrogenic chemicals.

Environmental estrogens, including endogenous estrogen, regulate estrogen-responsive genes such as VTG by binding to a specific estrogen receptor (ER); the estrogen-ER complex then interacts with the estrogen-responsive elements (EREs) in the target promoter genes to modulate their transcriptional activity. Furthermore, VTG induction is suppressed by treatment with estrogen antagonists in the presence of estrogen or estrogenic chemicals;[7] for example, egg shell protein choriogenin, which is regulated thorough estrogen action similar to VTG induction, is altered by estrogen or estrogenic chemical treatment.[8–11] Therefore, estrogen apparently drives the induction of VTG through the ER signaling pathway, and VTG is a sensitive biomarker for detecting estrogenic and/or anti-estrogenic chemical activities.[12–14] There are several common *in vitro* and *in vivo* VTG assays using fish; *in vitro* assays typically use cultured liver cells and *in vivo* assays use whole fish bred in test solutions.[15–18] These assays use VTG mRNA or VTG protein as an index.[19–23]

It has recently been clarified that many fish have multiple VTG subtypes, such as VTG1 and VTG2, by genome analysis.[24, 25] However, there appear to be differences in VTG subtypes when using them as biomarkers for the assessment of estrogenic chemicals. Fujiwara *et al.* measured serum VTG protein subtype immunologically using VTG1- or VTG2-specific antibodies in mature female medaka and 17β-estradiol (E2)-treated medaka. According to their report, VTG1 and VTG2 levels in mature female medaka serum were 13.02 ± 0.95 mg/ml and 1.63 ± 0.14 mg/ml, respectively, while in E2-treated male medaka, the levels were 13.40 ± 2.47 μg/ml and 8.97 ± 1.28 μg/ml, respectively.[26] They reported that VTG subtypes show differences in sensitivity and response to E2. Thus, it is necessary to assess VTG induction levels and sensitivity when investigating estrogenic activity and estrogenic chemical potency.

ER plays an important role in VTG induction. It has been suggested that fish have at least two ER subtypes, ERα and ERβ, and these genes are expressed in the liver, where VTG is synthesized.[27–29] Therefore, functional analysis and the effects of estrogenic chemicals need to be assessed for each ER subtype in fish; however there are currently few such studies.[30–32] Participation of ERα and ERβ VTG1 and VTG2 synthesis in fish, and the specific functions of ERα and ERβ in vitellogenesis are thus unclear. General VTG assays that do not distinguish VTG subtypes are not able to accurately characterize whether estrogenic or inhibitory action involves ERα and ERβ. Functionally distinguishing ERα and ERβ in vitellogenesis is required in order to understand the estrogenic and anti-estrogenic actions of endocrine-disrupting chemicals. Malamas *et al.* reported that 2-(4-hydroxyphenyl)-5-hydroxy-1,3-benzoxazole (HPHB) is an ERβ-selective ligand. HPHB show comparatively high selectively for ERβ, and weak affinity for ERα (ERα, IC_{50} = 1227 ± 533 nM; ERβ, IC_{50} = 49 ± 14 nM).[33] On the other hand, novel E2 analogs were synthesized by Kato,[34] however, they have never been tested for the affinity against ERα and β.

In this paper, we synthesized and screened E2 analogs as ERα-selective ligands, and synthesized ERβ-selective ligand. We then measured VTG1 and VTG2 mRNA induction in male medaka liver after treatment with ERα- or ERβ-selective ligands in order to investigate the characteristics of ER subtype function in VTG1 and VTG2 induction.

MATERIALS AND METHODS

Test Chemicals —— E2 (purity > 98%) and tamoxifen ([Z]-1-[*p*-dimethylaminoethoxyphenyl]-1,2-diphenyl-1-butene, purity > 99%) were obtained from Sigma (St. Louis, MO, U.S.A.). (17α, 20E)-3-hydroxy-17,20-[(1-methoxyethylidene)bis(oxy)]-19-norpregna-1,3,5(10),20-tetraene-21-carboxylic acid, methyl ester (orthoester 2k) and HPHB were synthesized and purified as described previously.[33, 34] HPHB was previously shown to have high ERβ selectivity.[33] Reagents were dissolved in dimethyl sulfoxide (DMSO, purity > 99%; Wako Pure Chemical Industries, Ltd., Osaka, Japan) for preparation of test solutions.

Yeast Two-hybrid Assay —— Assay for determining the estrogenic effects of test chemicals was performed as described previously.[35] Yeast cells (*Saccharomyces cervisiae* Y190) were modified by incorporation of human ERα (hERα) or hERβ, an expression plasmid with coactivator Translation initiation factor (TIF2), and a β-galactosidase expression reporter in a yeast two-hybrid assay.[36] The exposure concentrations of orthoester 2k (concentration ranges tested: 0.36, 0.73, 1.5, 2.9, 5.8, 11.7 and 23.3 μM), and E2 (31–2000 pM) were selected based on the results of a previous study.[37] All experiments were performed in triplicate for each compound.

Animals —— Medaka (d-rR strain) have been kept in glass tanks for several years in our laboratory. Male medaka are fed a diet of *Artemia* nauplii once daily. The tanks maintain a 16 : 8 light : dark photoperiod and a temperature of 25 ± 1°C. Male medaka (approximately 4 months after hatching) were exposed to test compounds as reported previously.[38] Test compounds were prepared as stock solutions dissolved in DMSO. Stock solutions were diluted with ion-exchange water to nominal concentrations and were mixed with a sonicator. Treatment concentrations were determined based on the results of two-hybrid assay; compounds were used at concentrations that showed activity equivalent to E2 3.7 nM on yeast two-hybrid assay.

Short-term Treatment with Orthoester 2k, HPHB and Tamoxifen —— Test solutions were prepared at several concentrations as follows: E2, 37 pM, 0.37 nM, 3.7 nM and 37 nM; tamoxifen, 1 μM and 10 μM; orthoester 2k, 2.8 nM, 28 nM, 280 nM and 2.8 μM; HPHB, 0.1 μM, 1 μM and 10 μM. Male medaka were then treated with test compounds, both alone and together with E2, at the above-mentioned concentrations. Chemical treatments were performed in 300-ml glass beakers at 25 ± 1°C under light or dark conditions. In exposure experiments, the control group was only exposed to 0.01% DMSO (solvent) for 8 hr. None of the exposure groups, including controls, were fed during the exposure period. At the end of the exposure period, livers were collected, rapidly frozen in liquid nitrogen, and stored at −80°C until analysis.

Long-term Treatment with Orthoester 2k, HPHB and E2 —— Male medaka were treated with following nominal concentrations: HPHB, 100 μM; orthoester 2k, 28 nM. Control fish were treated with DMSO. All treatments were carried out in a 2-l glass tank, and water was exchanged every other day. Under treatment, medaka were fed *Altemia* nauplii once daily, and water temperature was maintained at 25 ± 1°C with a 16 : 8 light : dark photoperiod. Treatment was carried out for 2, 4, 6 or 8 days, and at the end of the treatment period, livers were removed and rapidly frozen in liquid nitrogen, followed by storage at −80°C until analysis.

Quantitative Real-time PCR —— Total RNA preparation and reverse transcription were performed as described previously.[38] Quantification of hepatic VTG1, VTG2 and β-actin mRNA was performed by real-time PCR using FullVelocity SYBR Green Master Mix (Stratagene, La Jolla, CA, U.S.A.) and STRATAGENE Mx3000P™ (Stratagene). Specific primers for estrogen-responsive and reference genes were as follows: VTG1 (DDBJ accession no. AB064320) forward, 5′-TGGAAAGGCTGATGGGGAAG-3′, and reverse, 5′-AACTGCAGGCATGGTGAGCC-3′; VTG2 (AB074891) forward, 5′-GTCTTCAGGAGGTCTTCTTC-3′, and reverse, 5′-GGTAGACAATGGTATCCGAC-3′; β-actin (S74868) forward, 5′-AGACCACCTACAGCATC-3′, and reverse, 5′-TCTCCTTCTGCATTCTGTCT-3′. Reaction mixtures were incubated at 94°C for 5 min, followed by 35 PCR cycles at 94°C for 30 s, 62°C for 30 s and 72°C for 1 min. All experiments were performed in duplicate. Expression levels of VTG1and VTG2 mRNA were normalized against those of β-actin mRNA.

Statistical Analysis —— All experiments were performed using three medaka, and were repeated at least twice. Statistical analysis for VTG1and VTG2 mRNA expression was performed by one-way analysis of variance (ANOVA) using Excel NAG statistical analysis (Numerical Algorithms Group Ltd., Oxford, U.K.).

RESULTS

Yeast Two-hybrid Assay

In this study, we synthesized and screened E2 analogs as ERα-selective ligands[34] by using yeast two-hybrid assay incorporating hERα or hERβ. Among the analogs, orthoester 2k was shown highest selectivity for ERα. Chemiluminescence of recombinant hERα yeast cells increased depending on orthoester 2k concentration [Fig. 1 (A)]. However, in recombinant hERβ yeast cells, chemiluminescence was not detected [Fig. 1 (B)]. Thus, orthoester 2k showed specific affinity for hERα *in*

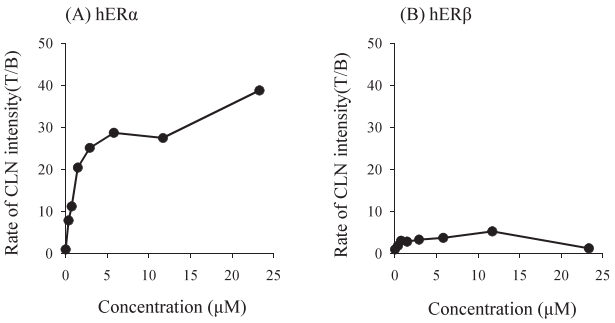

Fig. 1. Dose-response Curves for Orthoester 2k in Yeast Two-hybrid Assay with Recombined hERα (A) or hERβ (B)
Values indicate the ratio of chemiluminescence (CLN) intensity (T/B) *vs.* β-galactosidase.

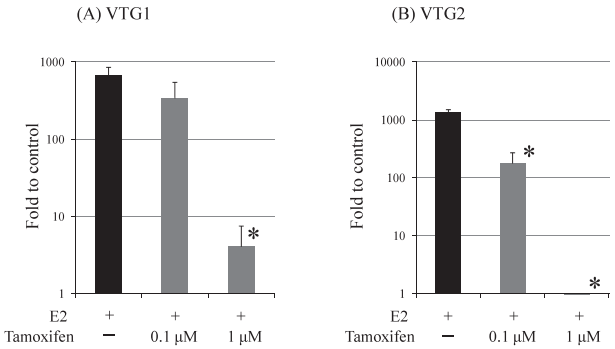

Fig. 2. Expression Levels of VTG1 and VTG2 mRNA in Male Medaka Liver after Exposure to ER Antagonist Tamoxifen in the Presence of E2 for 8 hr
Male medaka were treated with tamoxifen, both alone and together with 3.7 nM E2, at the indicated concentrations. Data are mean mRNA expression values ± S.D. (relative to β-actin mRNA expression, n = 3/dose). Asterisk (*) denotes significant differences *vs.* E2 treatment group ($p < 0.05$).

vitro [orthoester 2k Human ERα effective concentration $(EC)_{\times 10}$ = 2.8 μM; Human ERβ $EC_{\times 10}$ = Not Detected (ND)].

Tamoxifen Treatment

When male medaka were treated with E2 alone, VTG1 and VTG2 mRNA were both induced significantly. However, when treated with tamoxifen and E2, suppression of E2-induced increases in both VTG1 and VTG2 mRNA were noted. For VTG1 mRNA, significant suppression was not observed at 0.1 μM tamoxifen, but E2 induction was significantly suppressed by the presence of 1 μM tamoxifen [Fig. 2 (A)]. On the other hand, for VTG2 mRNA, induction was significantly suppressed even by 0.1 μM tamoxifen, and was completely blocked by 1 μM tamoxifen [Fig. 2 (B)]. These results suggest that both VTG subtypes are induced by E2, and that tamoxifen suppresses E2 induction. However, the responses of each gene to suppression by tamoxifen were different.

Short-term (8 hr) Treatment with Orthoester 2k or HPHB in the Absence of E2

We then determined the effects of ER subtype-selective agonists. VTG1 mRNA was induced by E2 treatment in a dose-dependent manner. Induction was increased by up to 400-fold when compared with control fish and was saturated at 3.7 nM E2. The ERα-selective ligand orthoester 2k induced VTG1 mRNA, even at low concentrations, under these conditions. Although VTG1 induction by orthoester 2k treatment was dose independent, maximum induction levels were equal to those of E2 treatment [Fig. 3 (A)]. On the other hand, the ERβ-selective ligand HPHB did not induce VTG1 mRNA at low concentrations. At high doses of HPHB, weak induction was observed. This induction may have been the result of the low affinity of HPHB for ERα [Fig. 3 (A)].

VTG2 mRNA induction was dose-dependently increased by 1500-fold with E2, as compared to negative control fish, and saturation was not observed. Orthoester 2k also induced VTG2 mRNA at all treatment points; however induction levels were less than half those of E2, even at maximum induction. HPHB also significantly induced VTG2 mRNA under all treatment conditions. At low concentrations, the induced levels were low but unambiguous induction was observed from 0.1 to 1 μM. At 10 μM HPHB, induction levels exceeded those with E2 alone [Fig. 3 (B)].

Taken together, these results show that VTG1 gene expression is mainly induced by E2-ERα complex. In contrast, VTG2 gene expression is regulated by both the E2-ERα and E2-ERβ complexes, but their relative contributions differ; the E2-ERα complex is necessary, but significant enhancement is mediated by the E2-ERβ complex.

Short-term (8 hr) Orthoester 2k or HPHB Treatment in the Presence of E2

Next, we conducted an experiment in order to clarify the relationship between E2 and ER ligands. Medaka treated with the ligands in the presence of 3.7 nM E2 induced VTG1 and VTG2 mRNA expression to 400-fold and 1500-fold levels when compared with control fish. In the presence of E2, additional induction was not observed with co-exposure to orthoester 2k. In the HPHB-treated group, VTG1 mRNA induction levels were lower than with E2 alone. Neither selective ligand showed additive or synergistic effects [Fig. 4 (A)]. These results confirmed that the VTG1 gene is regulated by

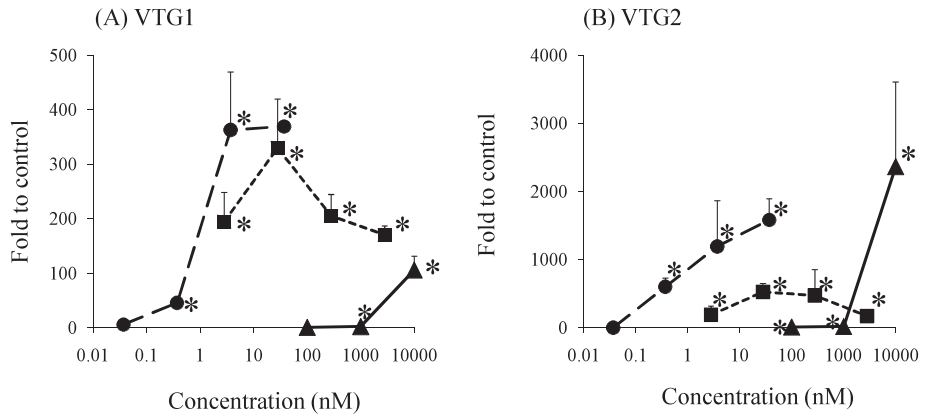

Fig. 3. Expression Levels of VTG1 and VTG2 mRNA in Male Medaka Liver after Exposure to E2 (●), ERα-selective Ligand (Orthoester 2k; ■), or ERβ-selective Ligand (HPHB; ▲) for 8 hr

Data are mean mRNA expression values ± S.D. (relative to β-actin mRNA expression, n = 3/dose). Asterisk (*) denotes significant differences *vs.* control group ($p < 0.05$).

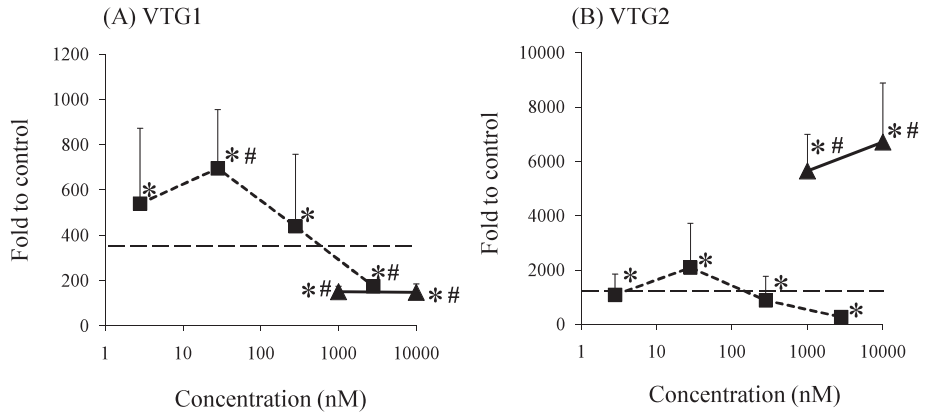

Fig. 4. Expression Levels of VTG1 and VTG2 mRNA in Male Medaka Liver after Exposure to ERα-selective Ligand (Orthoester 2k; ■) or ERβ-selective Ligand (HPHB; ▲) in the Presence of E2 for 8 hr

Dotted line refers to fold-change in VTG1 or VTG2 mRNA expression levels after treatment with 3.7 nM E2. Data are mean mRNA expression values ± S.D. (relative to β-actin mRNA expression, n = 3/dose). Asterisk (*) denotes significant differences *vs.* control group ($p < 0.05$). Sharp (#) indicates significant differences *vs.* E2 treatment group ($p < 0.05$).

the E2-ERα complex.

Orthoester 2k also had no effect on E2-induced VTG2 mRNA levels. On the other hand, on co-treatment with E2 and HPHB, substantial induction was observed in VTG2 mRNA levels [Fig. 4 (B)], and this was greater than that observed with HPHB or E2 alone [Fig. 3 (B)]. Furthermore, this induction was synergistic. For example, 10 μM HPHB alone showed slight induction of VTG2 mRNA, but VTG2 mRNA expression was induced at levels more than 3-fold higher on co-exposure to E2 and 10 μM HPHB.

Co-treatment with Orthoester 2k and HPHB

In order to confirm the function of ERα/β on VTG1/2 mRNA induction, we performed co-exposure to orthoester 2k and HPHB. Orthoester 2k at 2.8 nM induced weak VTG2 mRNA expression, while VTG2 mRNA induction with 0.1 μM HPHB did not differ from that in control groups. On the other hand, significant increase was observed with coexposure to 2.8 nM orthoester 2k and 0.1 μM HPHB. Large amounts of VTG2 mRNA were induced by co-exposure, and induction was 2-fold higher than with 2.8 nM orthoester 2k alone (Fig. 5).

Long-term Treatment with Orthoester 2k, HPHB and E2

Finally, we evaluated the effects of orthoester 2k or HPHB on estrogen responsive genes after 2–8 days of exposure. The concentrations used were 28 nM for orthoester 2k and 100 μM for HPHB. VTG1 mRNA was found to increase gradually over the exposure period, although HPHB showed

weaker effects than orthoester 2k, as HPHB has a lower ERα agonistic activity than orthoester 2k [Fig. 6 (A)]. On the other hand, HPHB increased VTG2 mRNA expression after 6-day exposure, but orthoester 2k did not [Fig. 6 (B)]. These results also indicate that VTG1 gene expression is ERα dependent, while VTG2 gene expression requires both ERα and ERβ.

DISCUSSION

VTG is a useful biomarker for detecting estrogenic activity of endocrine-disrupting chemicals in aquatic environments.[12–14] Our previous study demonstrated that expression patterns of VTG subtypes, such as VTG1 and VTG2, in the liver of male medaka are affected by treatment with estrogenic compounds, such as E2, nonylphenol and bisphenol A.[38] However, limited information is available on the regulation mechanisms of the expression of each VTG subtype, and the relationship between VTG1/2 and ER subtypes, such as ERα and ERβ. Soverchia *et al.* noted the possibility that ERβ is necessary for VTG induction, as increased plasma VTG protein and liver ERβ gene expression are concurrent.[30] On the other hand, Sabo-Attwood *et al.* reported that transcription of the ERα subtype is predominantly induced in concert with VTG in largemouth bass exposed to E2, and that ERβ subtypes depress ERα activation.[32] Moreover, Leaños-Castañeda and Van Der Kraak measured VTG induction in cultured liver cells from rainbow trout cells treated with ERα- or ERβ-selective ligands, and VTG induction was observed in ERβ-selective ligand-treated cells, but was not induced with the ERα-selective ligand.[31] To our knowledge, however, no information is currently available on the participation and functional distinction of ERα and ERβ in VTG1 and VTG2 synthesis after treatment with estrogenic compounds. Therefore, we investigated variations in VTG1 and VTG2 mRNA induction in male medaka liver treated with the ERα-selective ligand orthoester 2k, and the ERβ-selective ligands HPHB and tamoxifen using real-time PCR.

Tamoxifen is a known estrogen antagonist and is used as a therapeutic agent in breast cancer.[39] We evaluated the effects of tamoxifen against VTG subtype expression in order to obtain direct evidence of

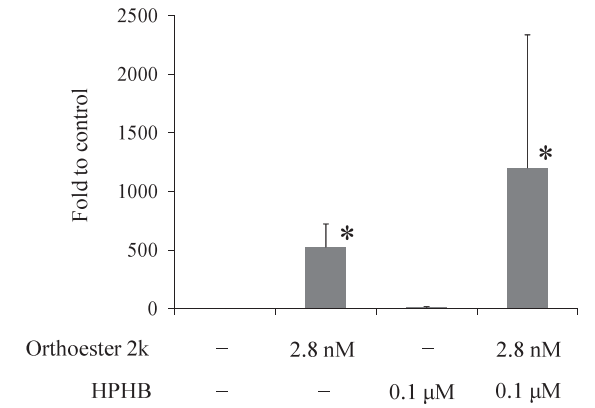

Fig. 5. Effects of ERβ-selective Ligand (HPHB) on Expression Levels of VTG2 mRNA in Male Medaka Liver after Exposure to ERα-selective Ligand (Orthoester 2k) for 8 hr

Data are mean mRNA expression values ± S.D. (relative to β-actin mRNA expression, n = 3/dose). Asterisk (*) denotes significant differences *vs.* control group ($p < 0.05$).

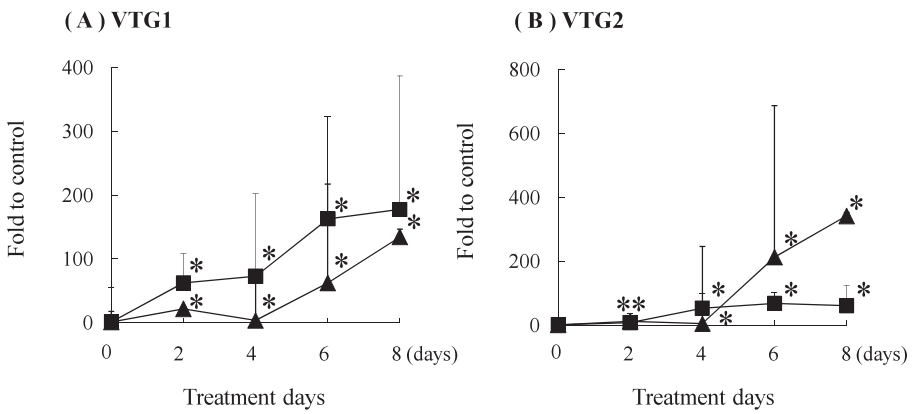

Fig. 6. Expression Levels of VTG1 and VTG2 mRNA in Male Medaka Liver after Exposure to ERα-selective Ligand (Orthoester 2k; ■) or ERβ-selective ligand (HPHB; ▲) for 2, 4, 6 or 8 Days

Data are mean mRNA expression values ± S.D. (relative to β-actin mRNA expression, n = 3/dose). Asterisk (*) denotes significant differences *vs.* control group ($p < 0.05$).

ER association. In the presence of E2, tamoxifen treatment showed a dose-dependent inhibitory effect on estrogen-responsive gene expression. However, the VTG1 and VTG2 inhibitory patterns were different, with VTG2 being more significantly affected by tamoxifen [Fig. 2 (A) and 2 (B)]. Based on these results, we speculated that both VTG1 and VTG2 gene expression are regulated through ERs, but the regulation mechanisms are different.

In order to investigate ERα and ERβ function, ERα- or ERβ-specific ligands are useful tools. We therefore synthesized several E2 analogs and surveyed the binding specificity for ERα or ERβ using yeast two-hybrid assay. Yeast two-hybrid assay is able to determine agonist or antagonist activity between test chemicals and to specify gene order.[36] The assay has also been used to determine the estrogenic activity of test chemicals.[37] Novel E2 analogs, including orthoester 2k, were synthesized by Kato,[34] and we investigated the affinity of each chemical for hERα and hERβ. Finally, we selected orthoester 2k as a novel ERα-specific agonistic ligand, as orthoester 2k was found to dose-dependently induce chemiluminescence on hERα yeast two-hybrid assay [Fig. 1 (A)]. On the other hand, on hERβ yeast two-hybrid assay, no chemiluminescence was observed [Fig. 1 (B)]. Malamas *et al.* reported that HPHB is an ERβ-selective ligand. HPHB show comparatively high selectively for ERβ, and weak affinity for ERα.[33] We also synthesized and used HPHB as an ERβ-selective ligand.

We then carried out short- and long-term exposure experiments using ER subtype-selective agonists. Hepatic VTG1 mRNA was induced by the ERα-selective ligand orthoester 2k, at even low concentrations, after short-term exposure [Fig. 3 (A)], and the maximum increase was similar to that with E2. On the other hand, VTG2 mRNA also increased, but only slightly, and the levels were lower than with E2 under the same conditions [Fig. 3 (B)]. In the presence of E2, no additional increases were observed with orthoester 2k (Fig. 4). On exposure to high doses of orthoester 2k, E2 action was suppressed. These observations suggest that orthoester 2k may simultaneously act through ERα with E2. Under long-term exposure, orthoester 2k induced VTG1 mRNA, even low concentrations, but VTG2 mRNA was not significantly induced (Fig. 6). These results indicate that ERα regulates VTG1 and VTG2 gene expression, but that their relative influence is different; VTG1 is mainly regulated through ERα, but VTG2 is not.

In contrast to orthoester 2k, the ERβ-selective ligand HPHB did not induce VTG1 mRNA at low concentrations [Fig. 3 (A)], but significantly induced VTG2 mRNA under all treatment conditions [Fig. 3 (B)]. In the presence of E2, VTG1 mRNA induction was lower than with E2 alone [Fig. 4 (A)]; HPHB apparently inhibited the action of E2. On the other hand, VTG2 mRNA increased significantly and these increases were higher than with E2 treatment alone [Fig. 4 (B)]. Thus, the action of E2 on VTG2 was enhanced by addition of HPHB. On co-exposure with orthoester 2k and HPHB, VTG2 mRNA increased significantly (Fig. 5). On long-term exposure, HPHB did not affect VTG1 mRNA, but increased VTG2 mRNA in a dose-dependent manner (Fig. 6). These results suggest that ERβ does not participate in VTG1 gene expression, but is closely involved in VTG2 expression. Initially, VTG2 gene expression occurs through ERα, but is then enhanced by the action of ERβ. Sabo-Attwood *et al.* reported that transcription levels of the ERα subtype were predominantly induced in concert with VTG in largemouth bass exposed to E2.[32] Our short- and long-term exposure results confirmed their observations, as ERα-selective ligands significantly induced VTG1 levels. Leaños-Castañeda and Van Der Kraak reported that VTG was not induced with ERα-selective ligands, and that VTG induction was observed in ERβ-selective ligand-treated cells.[31] However, they did not measure VTG subtypes separately. Our data also showed that ERβ is a major regulator of VTG2 gene expression in the presence of little ERα agonistic activity. Nonetheless, several issues remain unresolved, as there are three estrogen signaling pathways in mammals,[40] and fish estrogen signaling may utilize similar pathways.

The functional distinctions of ERα and ERβ on fish vitellogenesis may be complex with regard to VTG1 and VTG2 synthesis, particularly *in vivo*. Further research is necessary in order to elucidate to process of vitellogenesis in detail.

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