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Effects of endocrine disrupting chemicals on the expression of CYP19 genes in zebrafish (*Danio rerio*) juveniles

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Abstract

Cytochrome P450 aromatase (CYP19) is the key steroidogenic enzyme responsible for conversion of androgens to estrogens which play a critical role in developmental sex differentiation and adult reproductive cycles in vertebrates. To evaluate the potential roles of endocrine disrupting chemicals (EDC) on reproductive physiology of fish, the influence of multiple classes of EDC on the transcript abundance of two CYP19 isoforms, CYP19A1 and A2, were investigated in zebrafish juveniles. The pharmaceutical, clofibrate, and the pesticide, atrazine, did not influence the expression of either CYP19 gene. Estrogenic compounds, nonylphenol (NP) and a pharmaceutical estrogen, ethinylestradiol (EE), strongly enhanced the expression of CYP19A2 gene in dose-dependent manner. Exposure to benzo[a]pyrene (BaP) significantly increased CYP19A2 transcript abundance. Furthermore, BaP when co-treated with EE partially suppressed EE-induced upregulation of CYP19A2. In contrast, the expression of CYP19A1 was basically resistant to EDC treatment although EE at high concentration (1–100 nM) downregulated its expression. These findings suggest that multiple classes of EDC may potentially perturb developmental and reproductive physiology in fish through differential transcriptional modulation of the CYP19 genes with the most evident disruption in neural tissue.

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Keywords: CYP19; Endocrine disrupting chemical; Reproductive endocrinology; Zebrafish

1. Introduction

It has been widely recognized that estrogens, especially estradiol-17 β (E₂), play important roles in the development, growth, sexual differentiation and reproduction in all vertebrates studied to date. In lower

vertebrates, E₂ directly influences a number of developmental and reproductive events including, hepatic vitellogenin (the precursor of yolk protein) and egg membrane protein synthesis, oocyte growth (Wallace, 1985), germ cell development (Billard, 1992), gonadal sex differentiation (Jeyasuria et al., 1994; Wibbels et al., 1998), and reproductive behavior (Bjerselius et al., 2001). It is clear that appropriate and timely changes in E₂ biosynthesis are required for reproductive success. Alteration in the expression of genes encoding steroidogenic enzymes could negatively alter E₂ production.

Abbreviations: EDC, endocrine disrupting compounds; NP, nonylphenol; BaP, benzo[a]pyrene; EE, ethinylestradiol; CYP19, cytochrome P450 aromatase

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Cytochrome P450 aromatase (CYP19) is a crucial steroidogenic enzyme catalyzing the final, rate-limiting step in the conversion of androgens to estrogens. In mammals, with the exception of pig (Corbin et al., 1999), there is a single CYP19 gene that is expressed in a variety of tissues, is mediated through tissue-specific promoters and is characterized by alternative splicing of transcripts (Simpson et al., 2002). Teleosts, in contrast, have two structurally distinct CYP19 isoforms which share only 60% identity and are the products of different CYP19 gene loci, CYP19A1 and CYP19A2.¹ These gene products have been identified in several species of teleosts, including goldfish (Tchoudakova and Callard, 1998), zebrafish (Trant et al., 2001; Kishida and Callard, 2001), tilapia (Kwon et al., 2001) and catfish (Kazeto et al., unpublished): GenBank accession no. AF417239). CYP19A1 is predominantly expressed in the ovary and plays important roles in sex-differentiation and oocyte growth, whereas neuronal tissues, such as the brain, retina and pituitary, express CYP19A2 which is thought to be involved in the developing central nerve system (Kishida and Callard, 2001) and sex behaviors (Bjerselius et al., 2001). In addition, the involvement of the “brain aromatase” on reproductive physiology through the brain–pituitary–gonadal-axis has been suggested in fish (Cavaco et al., 2001; Kazeto et al., unpublished).

In recent years, considerable research effort has been focused on man-made chemical compounds that can modulate and/or disrupt the endocrine system in vertebrates (i.e. endocrine disrupting chemicals, EDC), including teleosts (Colborn et al., 1993, Sumpter, 1998). EDCs belong to diverse chemical classes and include pharmaceutical chemicals, herbicides, pesticides, industrial chemicals (e.g. plasticizer, polystyrenes, polychlorinated biphenyls), aryl hydrocarbons, and non-ionic surfactants and its decomposition products (alkylphenolic compounds), etc. Fish are considered an appropriate model for

EDC research because their environment is often exposed to multiple sources of EDCs, such as sewage, industrial effluent, and urban and agricultural runoff. There are numerous available reports on the effects of EDC on vitellogenin and CYP1A that are widely used biomarkers for xenoestrogens and aryl hydrocarbons, respectively (Rotchell and Ostrander, 2003). However, the molecular effects of EDC on the expression of other genes related to reproduction, including steroidogenic enzymes, have received little attention so far.

Recently, a number of consensus transcriptional regulatory elements were identified in the 5′-flanking regions of zebrafish CYP19A1 and CYP19A2 genes (Kazeto et al., 2001) that are responsive to putative EDC, which suggests that CYP19 genes would make excellent transcriptional targets for endocrine disruption. This report focuses on the aryl hydrocarbon responsive element (AhR/Arnt), estrogen responsive element (ERE) and a peroxisome proliferator-activated receptor α /retinoid X receptor α heterodimer responsive element (PPAR α /RXR α).

It has been demonstrated in zebrafish juveniles that E₂ upregulated CYP19A2 gene expression but not CYP19A1 transcription (Kishida et al., 2001) which is consistent with the structure of their 5′-flanking regions in that EREs are present in the promoter of CYP19A2 gene but not in CYP19A1 (Kazeto et al., 2001). However, as reported by Kishida et al. (2001), the concentrations of E₂ (0.01–10 μ M) were relatively high and gene expression analysis was conducted on 100–200 embryos pooled together. Furthermore, the effect of xenobiotics other than xenoestrogens on CYP19A2 expression has not been documented. The development of a highly sensitive real-time quantitative RT-PCR (Trant et al., 2001) permits the determination of transcript abundances of multiple genes in individual juveniles. The aim of this study was to determine the mechanism by which multiple classes of EDC potentially affect the in vivo expression of CYP19 genes in zebrafish. Ethinylestradiol (a pharmaceutical estrogen), nonylphenol (an alkylphenol and putative xenoestrogen), benzo[a]pyrene (a polycyclic aromatic hydrocarbon (PAH) and an activator of AhR), clofibrate (a peroxisome proliferator) and atrazine (a pesticide) were used as model EDCs. We hypothesized that the two CYP19 genes would respond differentially to ex-

¹ Through discussions with Daniel Nebert (University of Cincinnati Medical Center) and David Nelson (University of Tennessee, Memphis), the accepted rules of nomenclature for the superfamily of cytochromes P450 dictate these designations but they should apply to the duplicated genes of the teleosts only. This nomenclature should be substituted for the previously published designations of CYP19a and CYP19b.

posure and would be excellent biomarkers for EDC exposure.

2. Materials and methods

2.1. Chemicals for exposure

Para-nonylphenol (NP, minimum 92%), 3,4-benzpyrene (benzo[a]pyrene:BaP, minimum 97%), 2-(*p*-chlorophenoxy)-2-methylpropionic acid ethyl ester (clofibrate, minimum 98%) and 17 α -ethynyl-1,3,5[10]-estratriene-3,17 β -diol (ethinylestradiol:EE, minimum 98%) were purchased from Sigma–Aldrich (St. Louis, MO). 2-Chloro-4-ethylamino-6-isopropylamine-1,3,5-triazine (atrazine, minimum 97%) was obtained from Supelco (Bellefonte, PA). Letrazole was obtained from Novartis (Basel, Switzerland).

2.2. Zebrafish juveniles and EDC exposure

Adult zebrafish (*Danio rerio*) were maintained following established guidelines (Westerfield, 1995) in recirculating 401 aquarium tanks at 28 °C, on a 14 h:10 h light:dark cycle. Fertilized eggs were harvested and juveniles were raised to 17 days post-fertilization (dpf) according to Trant et al., 2001. The period of 17–20 dpf was chosen to conduct the exposure experiments because this is the time when both CYP19s are stably expressed with low individual differences (Trant et al., 2001). By avoiding much of the natural variations seen in zebrafish during growth and development, we were able to precisely examine the effects of EDCs on CYP19 expression. Furthermore, this period of development is biologically important because it is just prior to beginning of sex differentiation (Uchida et al., 2002).

Six zebrafish juveniles at 17 dpf were exposed together in a glass beaker (Fisher Scientific, Pittsburgh, PA) containing 500 ml of water for 3 days to either DMSO vehicle (0.1%, v/v) as the control group or multiple classes of EDCs, specifically, NP (a putative xenoestrogen; 0.01–1 μ M), BaP (an aryl hydrocarbon; 0.1–10 μ M), clofibrate (a peroxisome proliferator; 0.01–1 μ M), atrazine (a herbicide; 4.6–460 nM), EE (a pharmaceutical estrogen; 0.1 pM–100 nM) and letrazole (a non-steroidal specific aromatase inhibitor; 10 μ M) dissolved in DMSO (0.1%, v/v). In order to

examine the anti-estrogenic effects of putative non-estrogenic EDC on CYP19 expression, 3-days exposure experiments were carried out using EE (0.01 nM) plus BaP (0.1–10 μ M) or clofibrate (0.01–1 μ M). In preliminary experiments, these concentrations of BaP and clofibrate were determined to be effective in inducing Ah-responsive and PPAR-responsive genes in adult zebrafish liver.

It was assumed that 3 days is sufficient time for EDC exposure to alter the transcription of the target genes. However, in order to exclude the effects of endogenous estrogens, a longer exposure of letrazole would be required to reduce endogenous estrogen production. For this experiment, a continuous 30-day exposure to letrazole was initiated immediately after fertilization of zebrafish embryo/juvenile.

In all experiments, 50% of the contaminated water was changed every day for the duration of the exposure period. Following the exposure, all the fish were immediately flash frozen in liquid nitrogen and kept individually in a FastPrep Green tube (Bio101, Vista, CA) at –80 °C prior to use. All exposure experiments were conducted two to three times.

2.3. Quantification of transcript abundance of CYP19s

Total RNA was extracted from individual whole juveniles using Trizol Reagent (Life Technologies Inc., Gaithersburg, MD) and a FastPrep homogenizer (Savant Instruments, Farmingdale, NY). The concentration of total RNA prepared from each juvenile was determined spectrophotometrically and 2–4 μ g of total RNA was reverse-transcribed using M-MLV reverse transcriptase (Life Technologies Inc.) after priming with a clamped oligo(dT) primer (Kumar et al., 2000). A fluorescent-based method of real-time, quantitative RT-PCR (TaqMan; PE Applied Biosystems, 1997) was employed to measure the transcript abundance of CYP19A1 and CYP19A2 in zebrafish juveniles. Determination of transcript abundance of both CYP19 genes was conducted in duplicate, normalized to the abundance of β -actin (referred to as ΔC_t), and calculated relative to the average ΔC_t of the respective gene within the control group (referred to as $\Delta\Delta C_t$). The fold change in transcript abundance is calculated as $2^{-\Delta\Delta C_t}$ for each sample and the resultant data are then statistically analyzed. The average fold changes

for the control samples are expected to be close to but not equal to 1. The detailed procedure and all the information on primers and probes used for the Taq-Man analysis are described elsewhere (Trant et al., 2001).

2.4. Statistics

Fold changes in the transcript abundances of CYP19A1 and A2 genes were log-transformed to normalize the data and then subjected to one-way analysis of variance followed by Fisher's protected least significant difference (PLSD) post-hoc test. Differences were considered significant at $P < 0.05$.

3. Results

All exposure experiments were repeated two to three times. Figures represent one trial with the mean fold change and statistical analyses for the other trials presented in the associated table.

Although it has been shown that gene expression of β -actin (and many other housekeeping genes) may change in the face of physiological challenges (e.g.

Sarmiento et al., 2000). Changes in C_t (number of PCR cycles required for the signal to surpass a set threshold) for actin transcript abundances in the 3-day trials were not statistically different and were well within the expected individual and procedural variance. The greatest difference in the average C_t was observed in the group exposed to the highest concentrations of BaP. This increase of 0.6 C_t represents a 34% decrease in the mean actin transcript abundance, however, this difference was not statistically significant. We feel confident that actin expression, at least in this study, was a useful internal standard for normalization of the CYP19 data.

Exposures to all chemicals in this study had no effect on survivorship (100% survival in the 3-day exposures and 80–90% survival in the 30-day exposures).

3.1. Effects of EDCs on CYP19A1 gene expression

The 3-day, short-term exposures to EDCs had no effect on the expression of CYP19A1 gene in zebrafish juveniles with the exception of the high concentrations of EE exposure (1–100 nM) which significantly downregulated CYP19A1 expression (Fig. 1).

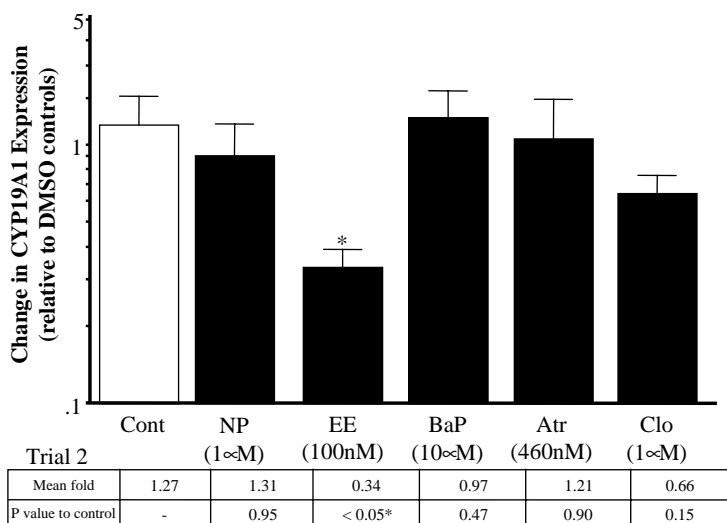


Fig. 1. Effects of multiple classes of EDC on CYP19A1 transcript abundance. Zebrafish juveniles at 17 dpf were exposed to NP, EE, BaP, atrazine (Atr) or clofibrate (Clo) for 3 days. Transcript abundance is expressed relative to that of the vehicle control group. Only the results from highest concentration of each exposure groups are shown since no alteration in CYP19A1 expression by lower concentration treatments were detected. The results represent the mean \pm S.E.M. of six samples. The asterisk indicates statistically significant differences ($P < 0.05$).

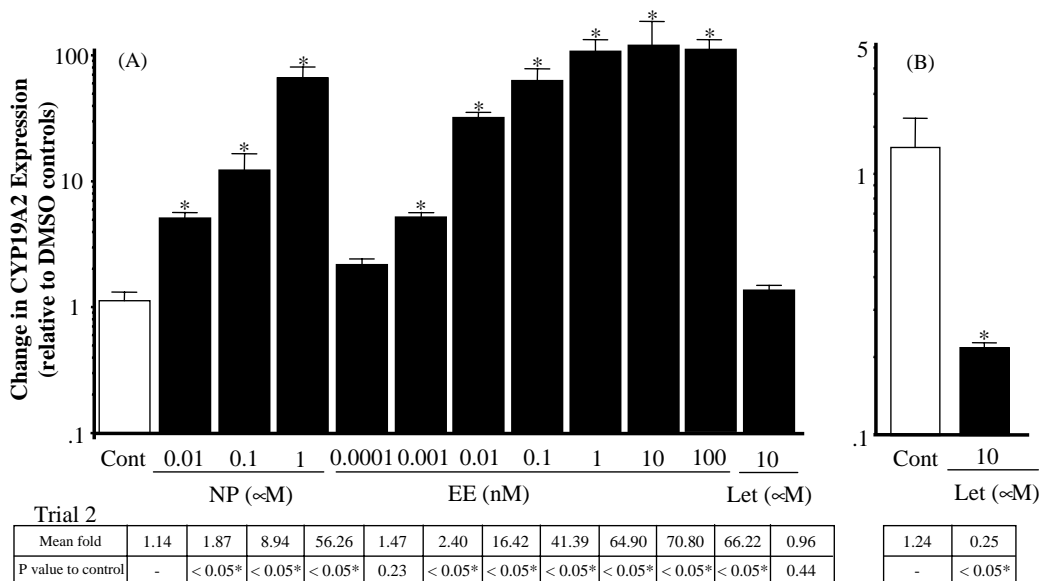


Fig. 2. Response of CYP19A2 transcription to the estrogenic chemicals, NP and EE, and an aromatase inhibitor, letrozole. CYP19A2 transcript abundance in zebrafish juveniles exposed to xenobiotics for 3 days (A) and 30 days (B). Transcript abundance is expressed relative to that of the vehicle control group. The results represent the mean \pm S.E.M. of six samples. The asterisk indicates statistically significant differences ($P < 0.05$).

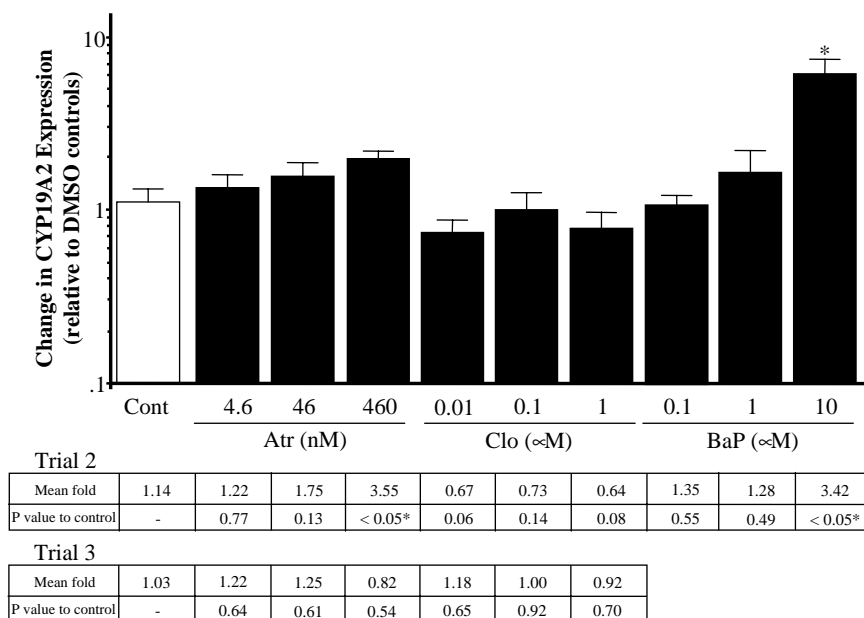


Fig. 3. Response of CYP19A2 transcription to BaP, atrazine (Atr) and clofibrate (Clo). Transcript abundance is expressed relative to that of the vehicle control group. The results represent the mean \pm S.E.M. of six samples. The asterisk indicates statistically significant differences ($P < 0.05$).

3.2. Effects of putative estrogenic chemicals and the aromatase inhibitor, letrozole, on CYP19A2 gene expression

Estrogenic compounds, EE and NP, strongly enhanced the expression of the CYP19A2 gene in a dose-dependent manner (Fig. 2A). Transcript abundance of CYP19A2 in the NP-exposed group at the highest concentration (1 μM) was approximately 60 times higher than that of the control group. NP treatment, even at the lowest concentration (0.01 μM), significantly increased CYP19A2 transcription. EE was the most potent modulator of CYP19A2 expression. Maximum upregulation of CYP19A2 gene expression, 60–100 times higher than controls, was induced by 1–100 nM EE exposure. Significant enhancement of its transcription by EE treatment was observed down to 0.001 nM in a dose-related fashion (Fig. 2A).

Long-term treatment (30 days) of zebrafish juvenile with the aromatase inhibitor, letrozole (10 μM), significantly reduced CYP19A2 transcript abundance although 3-day exposure failed to alter gene expression (Fig. 2B).

3.3. Effects of BaP, atrazine and clofibrate on CYP19A2 gene expression

The effects of non-estrogenic EDCs (BaP, atrazine and clofibrate) on CYP19A2 gene expression are shown in Fig. 3. CYP19A2 transcript abundance in the BaP exposed group at the highest concentration (10 μM) was approximately 5–10 times higher in trial 1 and three to four times higher in trial 2 than the control group. Slight or no influence of atrazine and clofibrate on CYP19A2 transcription was observed. Atrazine (460 nM) resulted in a significant three to four-fold increase in expression of CYP19A2 in only one concentration in one of three trials. In contrast, clofibrate did not modulate the expression of CYP19A2 gene significantly; however, there was a tendency for a decrease in abundance up to 60–70% in some cases.

3.4. Effects of BaP and clofibrate on EE-stimulated CYP19A2 transcription

In order to determine the influence of putative non-estrogenic compounds on EE-induced upregu-

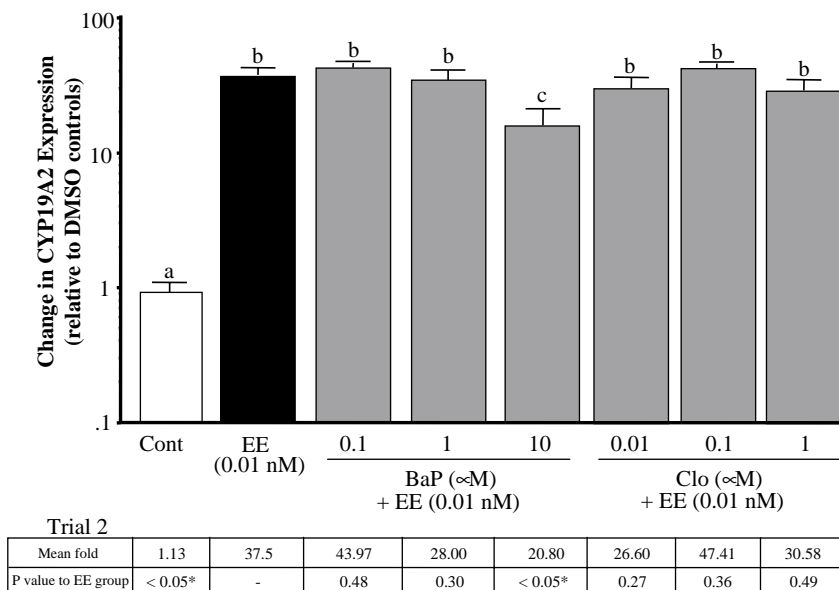


Fig. 4. CYP19A2 transcript abundance in zebrafish juveniles co-exposed to 0.01 nM EE and various concentrations of BaP or clofibrate (Clo) for 3 days. Transcript abundance is expressed relative to that of vehicle control group. The results represent the mean \pm S.E.M. of six samples. The asterisk indicates statistically significant differences ($P < 0.05$).

lation of CYP19A2 gene expression, co-exposures of EE (0.01 nM) and BaP (0.1–10 μ M) or clofibrate (0.01–1 μ M) were carried out. BaP at the highest concentration (10 μ M) partially blocked the stimulation of CYP19A2 transcription by EE up to 40–50%. However, clofibrate did not show any statistical anti-estrogenic effects on EE-induced CYP19A2 gene expression (Fig. 4).

4. Discussion

A number of potential responsive elements have been identified in the 5'-flanking regions of zebrafish CYP19A1 and CYP19A2 (Kazeto et al., 2001). Although some elements were expected (e.g. SF1 and CRE in the CYP19A1 gene), the presence of xenobiotic responsive elements was both surprising and important. The AhR/Arnt and PPAR α /RXR α in the promoter regions of the zebrafish CYP19 genes may serve as an excellent model to study the effects xenobiotic on sex steroid-mediated processes in larval development (e.g. sex differentiation) and reproduction (e.g. gametogenesis and behavior) if they can be shown to respond to exogenous compounds.

In the present study, the influence of multiple classes of EDC on the expression of two types of CYP19 genes in zebrafish juvenile was clearly established. The expression of CYP19A1, the predominant CYP19 in the gonad of most teleosts, was significantly suppressed by EE at high concentration but all other EDC treatments failed to modulate CYP19A1 expression in the zebrafish. Very recently, it has been reported that EE treatment causes strong suppression of early gonadal development in both male and female zebrafish (Hill and Janz, 2003; Weber et al., 2003), which may suggest that a decrease in CYP19A1 transcript abundance by EE treatment is not mediated directly through its 5'-flanking region but rather due to toxicity of xenoestrogens toward gametogenesis in this species. The potential binding site for an AhR/AhR nuclear translocation factor (AhR/Arnt) was identified in the 5'-flanking region of CYP19A1 gene of zebrafish (Kazeto et al., 2001) and goldfish (Tchoudakova et al., 2001). Furthermore, it was shown in rat granulosa cells that both the steady-state and FSH-stimulated mRNA levels of CYP19 are reduced by an AhR agonist,

2,3,7,8-tetrachlorodibenzo-*p*-dioxin, which is possibly attributable to the AhR/Arnt recognition sequence which is present in the promoter region of the gene (Dasmahapatra et al., 2000). Together these findings suggest that the possible modulation of CYP19A1 gene expression by agonists for AhR could not be ruled out in fish although BaP (an AhR agonist) treatment failed to alter CYP19A1 transcription in this report.

Transcription of CYP19A2, the aromatase isoform mainly expressed in the brain and pituitary, is strongly enhanced in zebrafish juveniles by a 3-day treatment with xenoestrogens, EE and NP. Upregulation of the transcript abundance or activity of aromatase expressed in the brain by estrogen has been demonstrated in some species of fish including goldfish (Gelinas et al., 1998), black porgy (Lee et al., 2000) and tilapia (Tsai et al., 2000). Our studies with zebrafish corroborate these findings.

Furthermore, long-term exposure to letrozole, an aromatase-inhibitor, reduced the transcript abundance of CYP19A2 though the 3-day exposure failed to alter the transcription. This difference between the results for short- and long-term exposure to letrozole may be due to the need of an exposure greater than 3 days to sufficiently inhibit estrogen synthesis and eliminate endogenous estrogens. Considering both the EE-induced upregulation and letrozole-induced downregulation of CYP19A2 transcription, it can be suggested that estrogen is one of main and comprehensive modulators of CYP19A2 expression in fish.

Recently, RT-PCR coupled with Southern blot analysis revealed a three to four-folds increase in CYP19A2 transcript abundance in zebrafish embryo/juvenile continuously exposed to E₂ (100 nM) for 2 days after fertilization (Kishida et al., 2001). However, we found that a similar treatment of zebrafish (EE treatment of 17 dpf juveniles at 1–100 nM for 3 days) resulted in nearly a 100-fold increase in transcript level of CYP19A2. This notable difference could probably be explained by the use of fish at different developmental stages and the different methods for quantifying gene expression. It is also noteworthy that in this study, the highly elevated levels of CYP19A2 expression were mediated by much lower and ecologically relevant concentration of EE (0.001–0.01 nM) and NP (0.01 μ M) than that described in the above report. These findings suggest

that the experimental design described here is highly sensitive in its ability to detect estrogenic potency of xenestrogens, e.g. NP and EE and CYP19A2 is a candidate gene to serve as a sensitive biomarker for exposure to environmentally relevant estrogenic compounds.

BaP was the only non-xenoestrogen examined in this study that could alter CYP19A2 expression, although its effectiveness was less and the effective concentration was much higher than that of xenoestrogens. In fact, the effective concentration of BaP was 100 times higher than environmentally relevant concentrations. The activators of AhR, including dioxins and polychlorinated biphenyls, are known to be accumulated in lipid-rich organs, including the brain, because of their hydrophobic nature (Ness et al., 1994; Wu et al., 2000). This implies that a better understanding of the potential role of CYP19A2 in AhR-mediated endocrine disruption may be provided with long-term, low concentration treatments with these chemicals. Furthermore, BaP at high concentrations may modulate CYP19A2 gene expression directly through the ERE because it has been shown that BaP and its metabolites may be a ligand for both the AhR and ER although the agonistic effect for ER is much lower than estrogen (Charles et al., 2000). The mechanism by which BaP modulates CYP19A2 transcription is still unresolved but this is the first report to demonstrate the stimulation of the CYP19A2 gene by a non-xenoestrogen.

Atrazine treatment at a high but environmental relevant concentration (460 nM) significantly increased transcript abundance of CYP19A2 in only one instance among the three trials. An additional exposure experiment using 1 and 10 mM of this chemical showed no significant difference in CYP19A2 transcript abundance (data not shown). The source of the variable response in the atrazine-treated group remains unclear but the increase of the transcript abundance does not seem to be due to atrazine but rather by uncontrolled factors, e.g. non-measured parameters of water quality, health of juvenile fish, differences in genetic backgrounds, etc. It is concluded that there is no significant effect of atrazine on CYP19A2 transcription.

Anti-estrogenic activity of the AhR-activators on the expression of several kinds of genes has been reported (Safe et al., 1998). The mechanisms of the inhibition of estrogen-induced responses remain un-

resolved but two different pathways may contribute to this anti-estrogenicity. One is through the rapid oxidative metabolism of E₂ by xenobiotic-metabolizing cytochromes P450 family of enzymes. Many of these gene families, including CYP1A, are strongly induced by AhR-activators in the liver. The other potential pathway is by suppressing transcription through the interaction of AhR/Arnt and ERE in E₂-responsive gene promoters (Safe et al., 1998). There is substantial evidence that CYP1A gene expression is inducible in brain and pituitary (Ortiz-Delgado et al., 2002; Rees et al., 2003; Iba et al., 2003), similar to that seen in hepatic tissues. Studies in our lab have clearly shown that the exposure of adult zebrafish to BaP will induce an upregulation of CYP1A in liver, brain and ovarian tissues (data not shown), therefore it is likely that other AhR-responsive, steroid metabolizing CYP genes could respond similarly.

Furthermore, EE, a 17 α -ethynylated form of E₂, would be inactivated by these steroid metabolizing CYPs since these enzymes mainly catalyze the hydroxylation of A-ring of E₂ (Badawi et al., 2000) whose structure is completely identical to that of EE. It is possible that a decrease in local concentrations of estrogen, due to a BaP-induced increased rate of estrogen degradation, could remove the inducer of CYP19A2 transcription in the brain and/or pituitary. However, we cannot rule out the possibility that BaP's anti-estrogenicity was a reflection of acute toxicity by BaP exposure at high concentration.

There is another potential mechanism explaining BaP's ability to up and downregulate CYP19A2 expression. BaP can act as a weak agonist for ER (Charles et al., 2000) to slightly upregulate CYP19A2 expression when acting on its own (Fig. 3) but it may act as an antagonist to the ER when in competition with estrogens thereby partially blocking estrogen's effect. The mechanism of anti-estrogenicity of ligands for AhR could be very complicated and further study would be necessary for better understanding of this phenomena.

In conclusion, estrogenic EDCs and BaP enhanced gene expression of CYP19A2 but not CYP19A1 in zebrafish juveniles. In addition, BaP showed potential anti-estrogenicity in estrogen-induced responses of CYP19A2 transcription. Therefore, some classes of EDC may potentially disrupt reproductive events of

the brain–pituitary–gonadal-axis through an alteration of CYP19A2 expression in fish without a direct effect on the CYP19A1 gene in the gonads.

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