Expression of Zebra Fish Aromatase cyp19a and cyp19b Genes in Response to the Ligands of Estrogen Receptor and Aryl Hydrocarbon Receptor

Ksenia Cheshenko,*,1 Francois Brion,†,1 Yann Le Page,‡ Nathalie Hinfray,† Farzad Pakdel,‡ Olivier Kah,‡ Helmut Segner,§ and Rik I.L. Eggen*†

*Eawag, Swiss Federal Institute of Aquatic Science and Technology, 8600, Dübendorf, Switzerland; †Unité d’évaluation des risques écotoxicologiques, Direction des Risques Chroniques, Institut National de l’Environnement Industriel et des Risques, BP 2, F-60550 Verneuil-en-Halatte, France; ‡Endocrinologie Moléculaire de la Reproduction, UMRCNRS 6026, Campus de Beaulieu, Université de Rennes 1, 35042 Rennes Cedex, France; and §Centre for Fish and Wildlife Health, University of Bern, Länggass-strasse 122, 3001 Bern, Switzerland

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Many endocrine-disrupting chemicals act via estrogen receptor (ER) or aryl hydrocarbon receptor (AhR). To investigate the interference between ER and AhR, we studied the effects of 17β-estradiol (E2) and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on the expression of zebra fish cyp19a (zf cyp19a) and cyp19b (zf cyp19b) genes, encoding aromatase P450, an important steroidogenic enzyme. In vivo (mRNA quantification in exposed zebra fish larvae) and in vitro (activity of zf cyp19-luciferase reporter genes in cell cultures in response to chemicals and zebra fish transcription factors) assays were used. None of the treatments affected zf cyp19a, excluding the slight upregulation by E2 observed in vitro. Strong upregulation of zf cyp19b by E2 in both assays was downregulated by TCDD. This effect could be rescued by the addition of an AhR antagonist. Antiestrogenic effect of TCDD on the zf cyp19b expression in the brain was also observed on the protein level, assessed by immunohistochemistry. TCDD alone did not affect zf cyp19b expression in vivo or promoter activity in the presence of zebra fish AhR2 and AhR nuclear translocator 2b (ARNT2b) in vitro. However, in the presence of zebra fish ERα, AhR2, and ARNT2b, TCDD led to a slight upregulation of promoter activity, which was eliminated by either an ER or AhR antagonist. Studies with mutated reporter gene constructs indicated that both mechanisms of TCDD action in vitro were independent of dioxin-responsive elements (DREs) predicted in the promoter. This study shows the usefulness of in vivo zebra fish larvae and in vitro zf cyp19b reporter gene assays for evaluation of estrogenic chemical actions, provides data on the functionality of DREs predicted in zf cyp19 promoters and shows the effects of cross talk between ER and AhR on zf cyp19b expression. The antiestrogenic effect of TCDD demonstrated raises further concerns about the neuroendocrine effects of AhR ligands.

Key Words: endocrine disruption; aromatase CYP19; zebra fish estrogen receptor; aryl hydrocarbon receptor; gene expression regulation.

In recent years, diverse cases of disturbed sexual differentiation and reproductive abnormalities have been reported in fish (Eggen et al., 2003; Jobling et al., 1998; Segner et al., 2003). These findings can be linked, at least partially, to exposure to so-called endocrine-disrupting chemicals (EDCs)—natural or synthetic compounds widely present in the environment that can disrupt hormone action. Aromatase P450, encoded by cyp19 gene(s), is considered to be a potential EDC target because it catalyzes the final step of biosynthesis of estrogens (Simpson et al., 2002), important hormones involved in the control of many physiological processes, including those related to reproduction. Several indications exist that interference with the aromatase CYP19 system in fish might lead to malfunctioning of the reproductive system. For instance, treatment with aromatase inhibitors disrupts gonadal sex differentiation in fish (Fenske and Segner, 2004). Two structurally distinct cyp19 genes, cyp19a1 (cyp19a) and cyp19a2 (cyp19b), are found in most teleosts, including model species zebra fish (Danio rerio). Zebra fish cyp19a (zf cyp19a) is predominantly expressed in gonads, and zebra fish cyp19b (zf cyp19b) is mainly found in neuronal tissues, while lower levels of the other isoform are found in both sites and in some other tissues (Chiang et al., 2001; Sawyer et al., 2006). Characterization of zf cyp19 promoters in zebra fish led to the assumption that some EDCs, for example, estrogen- and dioxin-like compounds, might interfere with the expression of these genes due to the presence of predicted responsive elements (Kazeto et al., 2001; Tong and Chung, 2003). Estrogen-responsive element (ERE) is found in the zf cyp19b promoter, half-EREs, and aryl hydrocarbon–responsive elements (also called dioxin-responsive elements, DREs) are predicted in...
Tanguay et al. The presence of ERE and DRE sites in the promoter points to potential regulation by respective receptors. Both estrogen receptor (ER) and aryl hydrocarbon receptor (AhR) function as ligand-dependent sequence-specific transcription regulators. Ligand-activated ER homodimer is able to initiate transcription from the promoters that possess a functional ERE (Klinge, 2000). Ligand-activated AhR heterodimerizes with AhR nuclear translocator (ARNT) and activates transcription of target genes through binding to DREs (Schmidt and Bradfield, 1996). Several isoforms of both ER and AhR/ARNT are found in teleost fish, including zebra fish (Andreasen et al., 2000). Ligand-activated ER homodimer is able to initiate transcription through binding to DREs (Schmidt and Bradfield, 1996). Several isoforms of both ER and AhR/ARNT are found in teleost fish, including zebra fish (Andreasen et al., 2000; Ohtake et al., 2003) effects of AhR ligands have been measured by a branched DNA assay (Quantigene, Genospectra, Fremont, CA, USA), as described previously (Hinfray et al., 2006). Briefly, for each exposure condition, two pools of 10 whole-body zebra fish larvae were constituted, lysed, and incubated in a 96-well plate coated with synthetic oligonucleotide in the presence of a specific probe set designed according to the zf cyp19a and zf cyp19b mRNA sequences (gene bank accession numbers AF183906 and AF183908, respectively). The probe set consisted of a capture probe that anchored the target mRNA to the synthetic oligonucleotide, a blocking probe that linearized the target mRNA, and of a label probe that hybridized to the target mRNA and to a branched DNA coupled with alkaline phosphatase–bound probes. Finally, a chemiluminescence substrate dioxetan that yields a luminescence signal proportional to the amount of mRNA present in the sample was added. Quantification of luminescence was made on a microplate luminometer (Wallac Victor2, Perkin Elmer, Courtabeuf, France). Zf cyp19a and zf cyp19b expression values were normalized to a housekeeping gene, zebra fish β-actin (gene bank accession number NM 131031). For each pool, measurements were performed in duplicate. The experiments were repeated three times on different days.

Immunohistochemistry. For each exposure condition, four zebra fish brains were analyzed. At the end of the exposure period, larvae were euthanized in MS-222, fixed in PBS (pH 7.4) containing 4% paraformaldehyde and 0.2% picric acid for 24 h at 4°C, and processed for cryosections (12 μm). Immunohistochemistry was performed as previously described (Mennet et al., 2005). Briefly, tissue sections were incubated overnight at room temperature with the polyclonal zebra fish CYP19B antibody (directed against the synthetic polypeptide CNSGETADNRTSKE corresponding to the last 15 residues of the protein sequence), diluted at 1:1000. Then the sections were rinsed and incubated with a biotinylated goat anti-rabbit IgG (1:1500) and a streptavidin-peroxidase complex (1:1500). Aromatase immunoreactivity was visualized to a housekeeping gene, zebra fish β-actin (gene bank accession number NM 131031). For each pool, measurements were performed in duplicate. The results were expressed as the fraction of positive sections and are the mean values of three replicates per exposure condition.

Cell culture. CHO-K1 (Chinese hamster ovary) and U251-MG (human astrocytes) cells were maintained at 37°C under 5% CO2 atmosphere in Dulbecco’s modified Eagle’s medium without phenol-red (DMEM; Sigma-Aldrich) supplemented with 100 U/ml of penicillin, 100 mg/ml of streptomycin, and 25 mg/ml of amphotericin (antibiotic-antimycotic solution, Sigma-Aldrich) and 9% inactivated fetal calf serum (PCS; Life Technologies, Carlsbad, CA). U251-MG medium additionally contained 2 mM of L-glutamine (Sigma-Aldrich).
Plasmid constructs used for transfection. The following plasmids were obtained from Dr. R. E. Peterson at the University of Wisconsin: the reporter plasmid pGL-1-luc, containing a dioxin-responsive promoter from the rainbow trout cyp1a gene (Abnet et al., 1999) in front of the luciferase-coding sequence, and the expression vectors zfAhR2 (Tanguay et al., 1999) and zfARNT2b (Tanguay et al., 2000), containing the full-length zebra fish AhR2 (zfAhR2) and ARNT2b (zfARNT2b)-coding sequences, respectively, in pBK-CMV. The expression vector zfERx containing the coding region of zebra fish ERx (zfERx) in Topo-pCDNA3 vector (Menuet et al., 2002). An empty Topo-pCDNA3 plasmid (Topo) containing no coding sequence for zebra fish receptors was used to equalize the amount of transfected DNA in the control (no exogenous receptor expression). The control estrogen-responsive reporter plasmid pERE-TK-luc contained an ERE site and a TATA box in front of the luciferase-coding sequence. The reporter plasmids cyp19b-luc and cyp19b_DREdel-luc (Menuet et al., 2005) contained — 486/+ 34 and — 371/+ 34 regions of cyp19b promoter/exon1 region in pGL-2-basic (Promega Corporation, Madison, WI, USA), respectively. Cyp19b_DREdel-luc was used as a DRE-deficient mutant (lacking predicted DRE sites with start positions — 453 and — 399).

The reporter plasmid cyp19a-luc contained — 536/+ 37 region of zfcyp19a promoter/exon1, cloned into pGL-2-basic vector. Total genomic DNA was obtained from adult zebra fish using conventional protocol (Nuesslein-Volhard and Dahm, 2002). The desired fragment was amplified by PCR from genomic DNA using a specific primer set designed according to the sequence of the Zfcyp19a promoter previously published (Kazeto et al., 2001), cyp19a-fw 5'-GGTACCATATGAAAGATGCGCTGAA-3', with nucleotide change (noted by lower case) introduced to create a KpnI site (underlined), and cyp19a-rev 5'-ACCTGGCATATAAGCAGGATGAGAGACAAAG-3'. The PCR reaction was carried out under the following conditions: 35 cycles consisting of denaturation at 95°C for 1 min, annealing at 65°C for 45 s, extension at 72°C for 45 s, and final extension at 72°C for 10 min. The PCR product was purified and cloned into pGEM-Teasy vector (Promega), then subcloned into pGL-2-basic vector with KpnI/SacI.

A QuickChange site-directed mutagenesis kit from Promega was used according to manufacturer's instructions to produce several mutated reporter constructs on the basis of cyp19a-luc. Gene Runner (Hastings Software Inc., Hastings-on-Hudson, NY, USA) was used to reexamine promoter regions. To create the cyp19a_DREmut-luc (mutated DRE site predicted at construct 37 region of zf-cyp19a promoter) (Menuet et al., 2005), cyp19a-fw 5'-GGTACCATATGAAAGATGCGCTGAA-3' (forward) was used (predicted site underlined, mutated bases denoted by lower case) introduced to create a KpnI site (underlined), and cyp19a-rev 5'-ACCTGGCATATAAGCAGGATGAGAGACAAAG-3'. The PCR product was purified and cloned into pGEM-Teasy vector (Promega), then subcloned into pGL-2-basic vector with KpnI/SacI.

In vitro translation and DNA-binding assays. ZfAhR2 and zfARNT2b proteins were produced from vectors zfAhR2 and zfARNT2b, respectively, in TNT rabbit reticulocyte lysate (Promega) according to supplier’s recommendations. Side reactions containing [35S]methionine were performed to assess relative protein production. After the 90-min incubation at 30°C, radioactive translation products were resolved on a 7.5% SDS polyacrylamide gel, dried, and detected on Hyperfilm MP (Amersham Life Sciences, Buckinghamshire, UK), exposed for 12 h. Unlabeled reactions were stored at —70°C prior to functional studies. The sequences of oligonucleotides used for DNA-binding assays are listed in Table 1. Oligonucleotide rt_DREfw was 5'-end labeled with [32P using T4 polynucleotide kinase and annealed to a three-fold molar excess unlabeled rt_DRErev to produce an rt_DRE probe, followed by purification. Unlabeled competitor DNAs were similarly produced by annealing unlabeled rt_DREfw/rt_DRErev, rt_a_160fw/rf_a_160rev, zf_a_238fw/rf_a_238rev, zf_b_222fw/rf_b_222rev, zf_b_399fw/rf_b_399rev, and zf_b_453fw/rf_b_453rev. An in vitro DNA-binding assay was performed essentially as previously described (Tanguay et al., 2000). Briefly, approximately equal amounts of in vitro produced zfAhR2 and zfARNT2b proteins were incubated in the presence of TCDD 10nM in DMSO 0.2% or DMSO 0.2% alone for 1 h at 22°C. Following incubation, 1.5 µg poly dI-dC and binding buffer (20mM HEPES, pH 7.9, 100mM NaCl, 1mM DTT, 6% glycerol) were added and the incubation continued for additional 20 min at 22°C before the addition of approximately 1 ng of the labeled rt_DRE probe with or without 50-fold molar excess of unlabeled rt_DRE, zf_a_160, zf_a_238, zf_b_222, zf_b_399, or zf_b_453 competitor oligos. Following 20 min incubation at 22°C, complexes were resolved on a 0.5% TBE (90mM Tris, 64.6mM boric acid, and 2.5 EDTA, pH 8.3) 4% acrylamide gel at 4°C. The signal was detected on Hyperfilm MP exposed for 12 h.

Statistical analysis. To evaluate in vivo exposure data, nonparametric ANOVA followed by Mann-Whitney U-test was used. The differences were considered significant at p < 0.05. For evaluation of in vitro data, a normalized luciferase activity was determined for each data point by dividing the luciferase activity by β-galactosidase activity and presented as a fold induction (mean ± SD) over control. The data were checked for normality (normal distribution of data). One-way ANOVA followed by Tukey Honestly Significant Difference Test was applied to analyze the differences.

### TABLE 1

<table>
<thead>
<tr>
<th>Oligo</th>
<th>Forward* sequence</th>
</tr>
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<tbody>
<tr>
<td>rt_DREfw</td>
<td>ACCTTTGAGCAGGCTACGAAAT</td>
</tr>
<tr>
<td>zf_a_160fw</td>
<td>ACACGTCGGAGGGTGGTATGGTGTAA</td>
</tr>
<tr>
<td>zf_a_238fw</td>
<td>AACTCGGAGCCGGGCAGATG</td>
</tr>
<tr>
<td>zf_b_222fw</td>
<td>GGAAAAAGATGTGGTATGGGCTAAT</td>
</tr>
<tr>
<td>zf_b_399fw</td>
<td>CGCTTATTGCTACGGCAACTTGT</td>
</tr>
<tr>
<td>zf_b_453fw</td>
<td>AATTAAGAAGCGGAATGAGGCCCCAC</td>
</tr>
</tbody>
</table>

*Reverse oligos were reversion complementary to forward oligos.

Note. Predicted DRE sites are underlined.

*Reverse oligos were reversion complementary to forward oligos.

*Disclaimer: The letters “a” or “b” correspond to zebra fish cyp19a or cyp19b genes from which the oligos were derived.

*The number indicates the position of start nucleotide of predicted DRE site in relation to exon start in the respective promoter.
between data points, which were considered significant at p < 0.01, unless otherwise noted.

RESULTS

Zfcyp19a and zfcyp19b Expression in Zebra Fish Larvae
In Vivo in Response to Exposure to Different Combinations of ER and AhR Ligands

No increased mortality was observed in zebra fish exposed for 3 days to 10nM E2 or to graded concentrations of TCDD or B[a]P alone or in combination with E2. Exposure to 10nM E2, 0.1nM TCDD, and 0.5μM ANF also was not toxic to the fish. However, the addition of 0.5μM ANF to 10nM E2 and 1μM B[a]P resulted in high mortality. Consequently, no data on the zfcyp19 expression could be obtained for this treatment condition. All measurements of zfcyp19 mRNA levels were performed on whole larvae extracts.

Exposure of zebra fish to 10nM E2 had no effect on zfcyp19a levels but significantly increased the expression of zfcyp19b (Figs. 1A and 1B). Exposure to graded concentrations of TCDD (0.001–0.1nM) or B[a]P (0.01–1μM) did not affect the expression of both zfcyp19 genes (Figs. 1A and 1B, only effect of highest concentration shown). Coexposure to 10nM E2 and 0.1nM TCDD (or 1μM B[a]P) had no effect on the zfcyp19a expression (Figs. 1A and 1B), regardless of whether the coexposure was done in the presence or absence of 0.5μM ANF (Figs. 1A and 1B). A different response was observed for zfcyp19b expression: the addition of 0.1nM TCDD or 1μM B[a]P to 10nM E2 led to significant downregulation of zfcyp19b expression in comparison with the expression induced by E2 alone (Figs. 1A and 1B). The downregulating effect of TCDD could partly be rescued by adding 0.5μM of ANF, a partial antagonist of AhR, to the mixture of E2 and TCDD (Fig. 1A). Furthermore, the changes in the CYP19B protein expression in the brain in response to exposure to several combinations of ER and AhR ligands were assessed by immunohistochemistry using zebra fish CYP19B antibody. In radial glial cells, 10nM E2 strongly induced CYP19B expression, but 0.1nM TCDD did not (Fig. 2). In fish, coexposed to 10nM E2 and 0.1nM TCDD, only a few CYP19B-positive cells were observed. In the group exposed to 10nM E2, 0.1nM TCDD and 0.5μM ANF, the staining was much more intense compared to the E2 + TCDD group, nonetheless, it was still less intense compared to the E2 group (Fig. 2). The expression pattern of CYP19A could not be assessed due to unavailability of specific antibody for this zebra fish protein.

Zfcyp19a and zfcyp19b Genes Promoter Activity In Vitro in Response to Treatment with Different Combinations of ER and AhR Ligands

To gain deeper insights into the effects of E2 and TCDD on zebra fish promoters activity, appropriate luciferase reporter gene assays were performed. The concentrations of treatment chemicals used in the in vitro experiments were below the cytotoxicity thresholds. The zfcyp19b promoter was studied in U251-MG (glial) cell line, previously reported as the cell context favorable for its activity (Menuet et al., 2005). Cyp19a-luc was initially transfected in CHO-K1 (ovarian) and U251-MG cells to assess the possible influence of the cell context on the promoter activity. Basal luciferase activity produced by cyp19a-luc in CHO-K1 was about 10 times higher than that in U251-MG. Moreover, treatment with 10nM E2 in the presence of zER2 led to upregulation of zfcyp19a promoter activity in CHO-K1 cell line, while no response was observed in U251-MG (Fig. 3). Subsequent studies of this promoter were performed in CHO-K1 cells only.
Comparing the response of zf\textit{cyp19a} promoter to E2 with and without cotransfected zf\textit{ER\alpha}, and using E2 antagonist ICI, it was possible to show that ER is needed for the upregulation of this promoter by E2 (Fig. 4). As a control, the empty pGL2-basic vector was subjected to the same analysis. Unexpectedly, it also responded to E2 treatment in the presence of zf\textit{ER\alpha} although the induction magnitude was lower (Fig. 4). Basal level of luciferase activity produced by cyp19a-luc in CHO-K1 cells was 26 ± 7 times higher than that produced by pGL2-basic. It was also higher (eightfold ± twofold) in comparison to cyp19b-luc in CHO-K1 cells. Furthermore, statistical analysis showed that the differences between the response of cyp19a-luc and pGL2-basic to E2 in CHO-K1 cells are significant ($\rho < 0.05$). This allowed us to presume that the response of cyp19a-luc to E2 in CHO-K1 cells is zf\textit{cyp19a} promoter specific. Since no ERE site is found in the zf\textit{cyp19a} promoter, we assumed that the indirect regulation by E2 might occur through the steroidogenic factor 1 (SF1) and half-ERE sites located in close proximity (SF1 at $-127$ bp and half-ERE at $-101$ bp from the transcription start). However, studies with mutated reporter gene constructs proved that this is not the case since the E2 response of constructs bearing mutations in the putative SF1 and half-ERE sites was not significantly different from that of the wild-type promoter (data not shown).

Next, the response of the zf\textit{cyp19a} and zf\textit{cyp19b} promoters to TCDD was investigated. The control dioxin-responsive promoter (prt1A-luc) exhibited dose-dependent response to TCDD treatment in the presence of zfAhR2/zfARNT2b in both CHO-K1 and U251-MG cells (Fig. 5; only one TCDD concentration [$10\text{nM}$] effect is shown). A slight upregulation in response to $10\text{nM}$ TCDD also occurred in the absence of zfAhR2/zfARNT2b. However, this effect was significantly

FIG. 2. CYP19B protein expression in the brain of zebra fish larvae (20 dpf) after 72 h exposure to DMSO 0.1% (DMSO), E2 10nM (E2), TCDD 0.1nM (TCDD), E2 10nM + TCDD 0.1nM (E2 + TCDD) or E2 10nM + TCDD 0.1nM + ANF 0.5μM (E2 + TCDD + ANF). Transverse sections (12 μm) were stained with zebra fish polyclonal CYP19B antibody. Four brains were analyzed per group; representative pictures are shown. No CYP19B immunoreactivity was observed in DMSO- or TCDD-exposed fish. Numerous CYP19B-positive radial glial cells were observed in the brain of E2-treated fish, while only few cells were stained in fish exposed to E2 + TCDD. The addition of partial AhR antagonist ANF to the E2 + TCDD partially restored the levels of CYP19B expression.
enhanced in the presence of these receptors (Fig. 5). The functionality of DRE sites predicted in the zf cyp19a and zf cyp19b promoters was examined using cyp19a-luc and its mutants, cyp19a_DREmut-luc and cyp19a_DREdel-luc, in CHO-K1 cells (Fig. 5A) and cyp19b-luc and its deletion
mutant, cyp19b_DREdel-luc, in U251-MG cells (Fig. 5B). The activity of the zf
\textit{cyp19a} promoter and its DRE-deficient mutants was not significantly affected by treatment with 10nM
TCDD in the absence of zfAhR2/zfARNT2b (Fig. 5A). However, the activity of the zf\textit{cyp19b} promoter was slightly
upregulated in the presence of TCDD and absence of zfAhR2/
zfARNT2b (Fig. 5B). The DRE-deficient mutant of zf\textit{cyp19b} promoter was upregulated similarly to wild type, and the E2-
responsive promoter containing just an ERE site and a TATA
box (pERE-TK-luc) responded to the TCDD treatment in the
absence of zfAhR2/zfARNT2b similarly to zf\textit{cyp19a} promoter
(Fig. 5B). The activity of zf\textit{cyp19a} and zf\textit{cyp19b} promoters, as
well as of the control E2-responsive promoter, was signifi-
cantly decreased in the presence of zfAhR2/zfARNT2b as
compared to activity without expressed exogenous receptors
(Fig. 5). The activity of the dioxin-responsive promoter was, on
the contrary, upregulated in the presence of zfAhR2/zfARNT2b and
absence of TCDD, and the addition of TCDD caused
further significant upregulation of this response (Fig. 5).
However, the addition of TCDD in the presence of zfAhR2/
zfARNT2b did not further affect the activity of zf\textit{cyp19b}
mutants or that of the control E2-responsive promoter as it
neither rescued the downregulation produced by expression of
zfAhR2/zfARNT2b nor did it cause further downregulating
effects (Fig. 5). The general response pattern of altered
constructs did not significantly differ from that of the wild

FIG. 6. The response of the zebra fish \textit{cyp19a}-luciferase reporter gene to


\textit{cyp19b}-luciferase reporter gene to
treatment with E2 or TCDD, alone or in combination, in CHO-K1 cells in the
presence of zebra fish ER\textalpha, AhR2, and ARNT2b. Cells were transfected with
the zf\textit{cyp19a}-luciferase reporter gene (zf\textit{cyp19a-luc}) and cotransfected with
a control vector (Topo) or vectors expressing zfER\textalpha, zfAhR2, and zfARNT2b.
Transfected cells were treated with DMSO (0.1%) with or without 10nM E2 or
1nM TCDD alone or in combination. The data are expressed as a fold induction
(mean ± SD) relative to control (Topo DMSO 0.1%) and represent a mean of
three independent experiments, where each point was performed in triplicates.
Data points significantly different from the control (Topo DMSO 0.1%) are
marked with “a” (p < 0.01).

FIG. 7. The response of different reporter genes to treatment with the ligands
of ER and/or AhR in U251-MG cells in the presence of zebra fish ER\textalpha, AhR2, and
ARNT2b in U251-MG cells. The cells were transfected with different reporter
genes (control estrogen-responsive promoter-luciferase [pERE-TK-luc],
zf\textit{cyp19b}-luciferase [zf\textit{cyp19b-luc}], and its DRE-deficient mutant [zf\textit{cyp19b_DRE-
del-luc}) and cotransfected with a control vector (Topo) or vectors expressing
zfER\textalpha, zfAhR2, and zfARNT2b. Transfected cells were treated with 10nM E2 or
1nM TCDD alone or in mixture or in combination with 1\textmu M ANF or 1\textmu M ICI.
The data are expressed as a fold induction (mean ± SD) relative to control (Topo DMSO
0.1%) and represent a mean of five independent experiments, where each point was
performed in triplicates. In (A), “a” indicates data points that are significantly
different from the control (Topo DMSO 0.1%); “b” indicates selected data points
that are significantly different from zf\textit{ER\textalpha}/AhR2/ARNT2b E2 10nM; and “c”
indicates selected data points that are significantly different from zf\textit{ER\textalpha}/AhR2/
ARNT2b TCDD 1nM. In (B), “a” indicates data points that are significantly
different from the control (Topo DMSO 0.1%); “b” indicates data
points that are significantly different from zf\textit{ER\textalpha}/AhR2/ARNT2b DMSO 0.1%
and “c” indicates data points that are significantly different from zf\textit{ER\textalpha}/AhR2/
ARNT2b TCDD 1nM. p < 0.01 in all cases.
competitor oligos derived from putative DREs in zf cyp19a. In vitro translated zfAhR2 and zfARNT2b proteins were incubated with 10nM TCDD and γ32P-labeled oligo rt_DRE derived from a DRE in the rainbow trout cyp1a promoter. In lane 2, 50-fold molar excess of unlabeled competitor oligo rt_DRE was added, and 50-fold molar excess of unlabeled competitor oligos derived from putative DREs in zf cyp19a and zf cyp19b promoters, zf_a_160, zf_a_238, zf_b_222, zf_b_399 and zf_b_453, was added in lanes 3–7. The experiment was repeated two times on different days, representative picture is shown. The arrow indicates the position of specific zebra fish AhR2/ARNT2b-rt_DRE complexes. The asterisk indicates the position of the free probe.

**FIG. 8.** Electrophoretic mobility shift assay of zebra fish AhR2 and ARNT2b interactions with DRE sites predicted in zebra fish cyp19a and cyp19b promoters. In vitro translated zfAhR2 and zfARNT2b proteins were incubated with 10nM TCDD and γ32P-labeled oligo rt_DRE derived from a DRE in the rainbow trout cyp1a promoter. In lane 2, 50-fold molar excess of unlabeled competitor oligo rt_DRE was added, and 50-fold molar excess of unlabeled competitor oligos derived from putative DREs in zf cyp19a and zf cyp19b promoters, zf_a_160, zf_a_238, zf_b_222, zf_b_399 and zf_b_453, was added in lanes 3–7. The experiment was repeated two times on different days, representative picture is shown. The arrow indicates the position of specific zebra fish AhR2/ARNT2b-rt_DRE complexes. The asterisk indicates the position of the free probe.

In the presence of zfERα, zfAhR2, and zfARNT2b, E2 response of zf cyp19a promoter activity was not significantly changed by the addition of 1nM TCDD (Fig. 6) or 1μM B[a]P (data not shown). However, coexposure to 10nM E2 and 1nM TCDD in the presence of zfERα, zfAhR2, and zfARNT2b led to significant downregulation of normal E2 response of the zf cyp19b promoter (Fig. 7A), which was rescued by the addition of 1μM ANF. In order to evaluate whether the effect of TCDD is dependent on the putative DRE sites present in the zf cyp19b promoter, we compared the responses of a wild-type zf cyp19b promoter and its DRE-deficient mutant. The two constructs exhibited the same general pattern of response to the treatments with ER and AhR ligands (Fig. 7A). The lower E2-induction magnitude of the cyp19b_DREdel-luc is due to deletion of half-ERE site upstream of the full ERE, as was shown previously (Menuet et al., 2005). Furthermore, we were able to show that a control E2-responsive promoter containing just an ERE site (pERE-TK-luc) also exhibited the same general response pattern to E2, TCDD, and ANF treatments (Fig. 7A).

TCDD alone led to slight but significant upregulation of the zf cyp19b promoter in vitro in U251-MG cells in the presence of zfERα, zfAhR2, and zfARNT2b (Fig. 7B). The addition of ER antagonist ICI (1μM) or AhR antagonist ANF (1μM) eliminated this slight upregulation (Fig. 7B). The DRE-deficient mutant of the zf cyp19b promoter and the control E2-responsive promoter exhibited the same general pattern of response to these treatments (Fig. 7B).

DNA-binding assays were performed to further investigate the ability of zebra fish AhR2/ARNT2b heterodimer to bind to the DRE sites predicted in the zf cyp19a and zf cyp19b promoters. Radioactively labeled probe rt_DRE designed on the basis of the rainbow trout cyp1a promoter (Tanguay et al., 1999) was used as a control. A strong complex was formed between the zfAhR2/zfARNT2b and rt_DRE probe in the presence of TCDD (Fig. 8, lane 1). This complex was reported to migrate as duplets of unknown nature (Tanguay et al., 2000). However, we have observed a single dispersed band migration. The complex was competed by a 50-fold molar excess of unlabeled rt_DRE (Fig. 8, lane 2), indicating that DNA binding is specific. None of the competitor oligos containing sequences of DREs predicted in zf cyp19a (zf_a_160, zf_a_238) and zf cyp19b (zf_b_222, zf_b_399, zf_b_453) promoters were able to compete with the active complex formation (Fig. 8, lanes 3–7).

**DISCUSSION**

The Effects of Estrogens on the zf cyp19a Expression

To establish the cellular system suitable for examining zf cyp19a promoter activity in vitro, two cell lines were transfected with a luciferase reporter driven by this promoter. Data demonstrated that the zf cyp19a promoter is more active in the ovarian cells context (CHO-K1) in comparison to glial cells (U251-MG). This finding corresponds well to in vivo observations since gonads (and especially ovary) were shown to be the main sites of zf cyp19a expression (Chiang et al., 2001; Fenske and Segner, 2004; Goto-Kazeto et al., 2004; Hinfray et al., 2006; Sawyer et al., 2006). We have observed a significant upregulation of the zf cyp19a promoter activity in E2-treated CHO-K1 cells in the presence of zfERα, in contrast to in vivo observations in zebra fish larvae where no upregulation of zf cyp19a by E2 was observed. In vitro E2 response was dependent on the presence of zfERα and also on the cellular context, as no upregulation was observed in U251-MG cells, even in the presence of zfERα. Surprisingly, the empty pGL2 basic vector, containing no promoter in front of luciferase-coding sequence, also responded to E2 treatment in the presence of zfERα, although the induction magnitude was significantly lower. Thus, although the observed induction of the zf cyp19a promoter by E2 may be an artifact induced by the empty vector, significantly higher basal luciferase activity and response to E2 of cyp19a-luc allowed us to presume that this
induction is zf\(cyp19a\) promoter specific. The discrepancy between our \textit{in vivo} and \textit{in vitro} observations on the zf\(cyp19a\) expression in response to E2 can be partially explained by the higher sensitivity of the \textit{in vitro} reporter gene assays compared to the \textit{in vivo} assay. Differences between the response of artificial reporter genes and endogenous target genes expression have been observed even in the same cell context (Shipley and Waxman, 2005). It is also possible that the induction of the zf\(cyp19a\) expression \textit{in vivo} occurs only in specific cell types; thus, this effect is masked when measurements are performed on the whole-body homogenates. Moreover, it is well known that response to estrogens depends on several factors other than simple ligand-ER binding, such as ligand transport to target tissues or bioactivation, which may add to differences between \textit{in vitro} and \textit{in vivo} estrogenic responses.

Our \textit{in vivo} results agree with previous studies that have shown the lack of estrogens’ effect on zf\(cyp19a\) expression in larvae (Hinfray et al., 2006). In another study, downregulation of zf\(cyp19a\) expression in larvae was observed in response to exposure to 1–100nM of EE2 (Kazeto et al., 2004), but the authors suggested that this effect is not mediated directly through the 5′-flanking region of zf\(cyp19a\). Exposure of adult female zebra fish to 10nM of E2 for 7 days also suppressed zf\(cyp19a\) expression and aromatase activity in the ovary (Hinfray et al., 2006). On the contrary, in the protandrous black porgy (Acanthopagrus schlegeli), treatment of undifferentiated fish with 6 mg E2/kg for 3 months resulted in an increased gonadal aromatase activity (Lee et al., 2004), but it was not established if the observed increase in aromatase activity was the result of enhanced expression of \(cyp19a\) or \(cyp19b\). Cyp19a mRNA levels in the gonad of \textit{Rivulus marmoratus} were upregulated in response to BPA, but downregulated by NP (Lee et al., 2006). Cyp19a expression was also upregulated in the brain of Atlantic salmon juveniles exposed to EE2 (Lyssimachou et al., 2006). Our results together with published data show that the estrogen effects on \(cyp19a\) expression may differ depending on the biological model used (\textit{species}, \textit{in vitro} \textit{vs.} \textit{in vivo}) as well as on the experimental design employed (life stage of development, mode of fish exposure, examined tissues, and ER ligand used). Further detailed investigation of \textit{in vivo} effects of estrogens on \(cyp19a\) gene expression throughout the course of development might be of interest.

Examination of the Functionality of DRE Sites Predicted in the zf\(cyp19a\) and zf\(cyp19b\) Gene Promoters

Exposure to TCDD had no effect on the zf\(cyp19a\) or zf\(cyp19b\) mRNA levels in zebra fish larvae \textit{in vivo}. We also examined the functionality of DRE sites predicted in zf\(cyp19\) promoters \textit{in vitro} using zf\(AhR2\) and zf\(ARNT2b\) receptor proteins. Zf\(AhR2\) was shown to be the functional receptor form both \textit{in vitro} and \textit{in vivo} (Prasch et al., 2003; Tanguay et al., 1999), Zf\(ARNT1\) splice proteins, but not zf\(ARNT2b\), were suggested to be the preferred dimerization partners for zf\(AhR2\) \textit{in vivo} (Prasch et al., 2004, 2006). However, zf\(ARNT2b\) was shown to form a functional heterodimer with zf\(AhR2\) \textit{in vitro} that can specifically recognize DREs and induce DRE-driven transcription (Tanguay et al., 2000), which justifies the use of this form of zebra fish ARNT in the present \textit{in vitro} assays. The activity of the control dioxin-responsive promoter was upregulated by TCDD (or B[a]P) treatment in the presence of zf\(AhR2/zfARNT2b\) in the CHO-K1 and U251-MG cells, which confirmed that these cellular systems contain all the co-factors necessary for the functioning of a classical AhR pathway. A slight upregulation in response to AhR agonists also occurred in the absence of zf\(AhR2/zfARNT2b\). However, significantly higher upregulation in the presence of zf\(AhR2/zfARNT2b\) suggests that the low levels of endogenous receptors expressed in CHO-K1 and U251-MG cells do not hinder the observation of specific effects of exogenously expressed receptors. In the absence of zf\(AhR2/zfARNT2b\), TCDD treatment did not affect zf\(cyp19a\) promoter activity. It did, however, slightly upregulate the activity of zf\(cyp19b\) promoter. The DRE-deficient mutant of this promoter was similarly upregulated under these conditions, suggesting the independence of the response observed from the predicted DRE sites. The possible mechanism of this upregulation is discussed in the next subsection. Expression of zf\(AhR2/zfARNT2b\) led to significant downregulation of the activity of zf\(cyp19a\) and zf\(cyp19b\) promoters, their DRE-deficient mutants, and of the control E2-responsive promoter containing only an ERE site. The addition of TCDD (or B[a]P) had no further effect on this downregulation. Thus, the downregulation of promoter activity caused by expression of zf\(AhR2/zfARNT2b\) seems to be an unspecific phenomenon, independent of DREs predicted in the zf\(cyp19a\) and zf\(cyp19b\) promoters. This could be due to the generally increased translation burden on the cells. Interestingly, the activity of the control dioxin-responsive promoter was significantly increased in the presence of zf\(AhR2/zfARNT2b\) even in the absence of TCDD, suggesting that functional DRE elements can promote higher basal activity of the promoter in the presence of high levels of AhR and ARNT, even without the ligand. In \textit{in vitro} DNA-binding assays, zf\(AhR2/zfARNT2b\) heterodimer binds to DRE even in the absence of ligand (Tanguay et al., 2000). Thus, it is possible that in the cell culture the exogenously expressed unliganded zf\(AhR2/zfARNT2b\) were not readily bound by histones and therefore were able to exert some transcriptional effects on the activity of promoters containing functional DREs. Overall, the pattern of zf\(cyp19a\) and zf\(cyp19b\) promoters’ response to zf\(AhR2\), zf\(ARNT2b\), and TCDD, which drastically differed from that of the control dioxin-responsive promoter, indicated the nonfunctionality of predicted DRE sites. We also demonstrated by electrophoretic mobility shift assays the inability of DREs predicted in the zf\(cyp19a\) and zf\(cyp19b\) promoters to bind to liganded zf\(AhR2/zfARNT2b\) heterodimer. The unresponsiveness of zf\(cyp19a\) and zf\(cyp19b\)
to TCDD may be due to low conservation of consensus sequence.

Observed unresponsiveness of zfcyp19a to AhR agonists stands in agreement with several reports (Hoffmann and Oris, 2006; Kazeto et al., 2004). However, diverse effects of AhR agonists on aromatase in the ovary were observed in other studies. In mice, AhR cooperates with SF1 to activate cyp19 transcription in ovarian granulosa cells (Baba et al., 2005). TCDD decreases cyp19 mRNA levels in cultured rat granulosa cells, but the dependence of this effect on DRE-like sites in the promoter was not established (Dasmahapatra et al., 2000). TCDD reduced ovarian aromatase activity in adult female zebra fish (King Heiden et al., 2006). B[a]P inhibited CYP19 in ovary tissue of flounder (Platichthys flesus) in vitro (Rocha Monteiro et al., 2000) and in adult female killifish ovary in vivo, without affecting cyp19a mRNA levels (Patel et al., 2006). Thus, exposure to AhR ligands apparently may have an effect on aromatase activity in the ovary, but in respect to an effect on cyp19a expression, the evidence is equivocal.

Exposure to B[a]P was shown to increase mRNA levels of cyp19b in zebrafish larvae in 3 days exposure assay as well as in adult females exposed from immature stage for 56 days, but it was not the AhR activation but rather the weak estrogenic activity that was suggested to be primarily responsible for this effect (Hoffmann and Oris, 2006; Kazeto et al., 2004). The lack of zfcyp19b induction by B[a]P in our assay might be due to the lower concentrations used than in the study by Kazeto et al. (2004).

Altogether, these data provide evidence that TCDD and B[a]P may possibly alter the expression and/or activity of cyp19 genes via several mechanisms, which might differ between different organisms. However, our data provide strong evidence that DRE sites predicted in the zfcyp19a and zfcyp19b promoters are not functional. Consequently, it is unlikely that either the isoform of the zfcyp19 genes can be used as a biomarker of exposure to dioxin-like compounds.

Cross Talk between ER and AhR and Its Effects on the zfcyp19b Expression

E2 strongly upregulated the activity of the zfcyp19b promoter in the reporter gene assays and the expression of zfcyp19b in zebrafish larvae, leading to de novo synthesis of CYP19B protein in radial glial cells, in agreement with previous studies (Hinfray et al., 2006; Kishida et al., 2001; Menuet et al., 2005). TCDD attenuated the normal E2-induced response of zfcyp19b expression. This was either partially (in vivo) or fully (in vitro) rescued by the addition of AhR antagonist, ANF, suggesting the involvement of AhR in the downregulation mechanism. The inability to observe full rescue in vivo could be explained by the lower ANF concentration used compared to in vitro experiments (due to observed toxicity of higher concentrations in combination with TCDD). It might also reflect the complexity of in vivo system setup, including the rates of uptake, metabolism and biodegradation of the chemicals, or the fact that ANF is a partial and not a full antagonist of AhR. Another AhR ligand, B[α]P, also downregulated the zfcyp19b response to E2 both in vivo and in vitro in our study and to EE2 in the other (Kazeto et al., 2004). The effect of cotreatment with ANF on the B[α]P-induced changes in the E2 response of zfcyp19b could not be measured because the exposure to E2, B[α]P, and ANF led to high mortality of the larvae, as was also observed by others (Billiard et al., 2006). However, in vitro observations suggested the involvement of AhR in this mechanism. Induced changes in zfcyp19b mRNA levels were closely paralleled on the protein level, thus confirming the functional significance of our findings on the effects of ER and AhR ligands on the zfcyp19b expression.

In our in vitro studies, TCDD downregulated the E2-induced response driven from a single ERE site on the promoter, independent of putative DRE sites or any other transcription sites, as was shown by experiments with the DRE-deficient mutant of the zfcyp19b promoter and with the E2-responsive promoter containing an ERE site, only. Similar results were recently obtained with mammalian receptors, showing that AhR agonists attenuate E2-induced transcription from a single ERE site, without binding directly to ERs or affecting expression levels of ERs (Ohtake et al., 2003). Evidence of a similar mode of action in teleosts has also been observed in another study (Bemanian et al., 2004).

Previous studies on the antiestrogenic effect of AhR ligands in fish focused mainly on hepatic E2 target genes involved in vitellogenesis (Anderson et al., 1996; Navas and Segner, 2000). To our knowledge, the present work provides the first report showing the antiestrogenic effect of an AhR agonist on an E2-regulated gene within a glial cell context. The biological significance of this effect is not known. However, radial glial cells are known to play a crucial role in embryonic and adult neurogenesis (Götz et al., 2002), and E2 is an important neurotrophic and neuroprotective factor. Further, it has been shown that AhR is expressed in the areas of fish brain involved in neuroendocrine regulation of reproductions, such as hypothalamus and gonadotropin cells (Ortiz-Delgado et al., 2002). Therefore, it can be hypothesized that disruption of the normal E2-induced expression of aromatase (and hence of local synthesis of E2) in radial glial cells may be a significant pathway for neuroendocrine effects of AhR ligands in vertebrate.

Exposure to TCDD or B[α]P alone had no effect on the zfcyp19b mRNA levels in zebrafish larvae or on the zfcyp19b promoter–driven luciferase expression in U251-MG cell line in the presence of zfAhR2/zfARNT2b in vitro. However, a slight upregulation of this gene’s promoter activity by TCDD was observed in the presence of zfERα, zfAhR2, and zfARNT2b proteins. This effect was blocked by cotreatment with an excess of either ER antagonist ICI or AhR antagonist ANF, suggesting the involvement of both ER and AhR in the process. Experiments with the DRE-deficient mutant of the zfcyp19b promoter...
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and the control E2-responsive promoter, containing one ERE site only, suggested that this mechanism is independent of predicted DRE sites, and only the ERE site is involved in this type of ER and AhR interaction. It was shown that liganded mammalian AhR/ARNT heterodimer can directly associate with unliganded ER, leading to stimulation of ERE-mediated transcription (Ohtake et al., 2003), in agreement with our present findings. In light of this, the slight upregulation of the zfcyp19b promoter activity and its DRE-deficient mutant as well as of the control E2-responsive promoter, observed in the presence of TCDD without any expressed zebra fish receptors, can be explained by the presence of low levels of mammalian ER, AhR, and ARNT, which were able to engage in the same kind of action. The differences between fold induction numbers observed in our study and the study of Ohtake et al. (2003) can be attributed to either the structural differences between mammalian and fish receptors, which may account for differences in relative potencies of ligands between mammals and fish (Abnet et al., 1999), or to the different cellular contexts used and the different promoters studied. We observed the weak estrogenic effect of TCDD in vitro only in the absence of the ER ligand, as was also shown by others (Ohtake et al., 2003). Thus, the inability to observe upregulation of endogenous zfcyp19b gene expression by TCDD in vivo in zebra fish larvae can be explained by the presence of endogenous estrogens, which preclude potential estrogenic actions of dioxins.

In summary, we have evaluated the effects of ER and AhR ligands on the activity of zfcyp19a and zfcyp19b genes by in vivo exposure of zebra fish larvae and by in vitro luciferase reporter gene assays. The zfcyp19a gene was not affected by treatment with E2 in vivo. However, a slight upregulation of this gene promoter was observed in vitro, the mechanism of which could not be clarified at the moment. In our study, AhR ligands, TCDD, or B[a]P, did not affect zfcyp19a and zfcyp19b expression in vivo, and in vitro experiments suggested the nonfunctionality of predicted DRE sites. However, we have shown the attenuation of normal E2-induced upregulation of zfcyp19b expression by AhR ligands both in vivo and in vitro. We confirmed the involvement of AhR in this mechanism and were able to show in vitro its independence from the putative DRE sites predicted in the zfcyp19b promoter. This inhibition of E2-induced zfcyp19b expression in radial glial cells is a novel mode of AhR ligands action, which points to the possible disruption of neuroendocrine functions of estrogens as one of the toxic effects of this compound. We have also observed a slight induction of zfcyp19b promoter activity by TCDD in vitro, which seems to function in accordance with the mechanism where liganded AhR/ARNT associates with unliganded ER and triggers transcription from the ERE site. Thus, here we show that in fish, as it has been demonstrated in mammals, the levels of ER agonist estrogen might determine the estrogenicity or antiestrogenicity of AhR agonists in relation to estrogen-responsive genes, and that ER/AhR cross talk follows several pathways, not always depending only on the presence of DRE sites in the gene promoters. These findings should be taken into account while interpreting the results of studies investigating the estrogen-related actions of AhR ligands, especially, in mixtures. Further investigation of interactions between ER, AhR, their ligands, and gene promoters, as well as characterization of coregulators of this process, might greatly facilitate the research on the estrogen-related actions of dioxins.

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