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Estrogen and aryl hydrocarbon receptor expression and crosstalk in human Ishikawa endometrial cancer cells

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Abstract

Ishikawa endometrial cancer cells express the estrogen receptor (ER), and this study investigates aryl hydrocarbon receptor (AhR) expression and inhibitory AhR–ER crosstalk in this cell line. Treatment of Ishikawa cells with the AhR agonist [^{3}H]2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) gave a radiolabeled nuclear complex that sedimented at 6.0 S in sucrose density gradients, and Western blot analysis confirmed that Ishikawa cells expressed human AhR and AhR nuclear translocator (Arnt) proteins. Treatment of Ishikawa cells with 10 nM TCDD induced a 9.7-fold increase in CYP1A1-dependent ethoxyresorufin *O*-deethylase (EROD) activity and a 10.5-fold increase in chloramphenicol acetyltransferase (CAT) activity in cells transfected with pRNH11c containing an Ah-responsive human CYP1A1 gene promoter insert (−1142 to +2434). Inhibitory AhR–ER crosstalk was investigated in Ishikawa cells using E2-induced cell proliferation and transcriptional activation assays in cells transfected with E2-responsive constructs containing promoter inserts from the progesterone receptor and vitellogenin A2 genes. AhR agonists including TCDD, benzo[a]pyrene (BaP) and 6-methyl-1,3,8-trichlorodibenzofuran, inhibited 32–47% of the E2-induced responses. In contrast, neither estrogen nor progesterone inhibited EROD activity induced by TCDD in Ishikawa cells, whereas inhibitory ER-AhR crosstalk was observed in ECC-1 endometrial cells suggesting that these interactions were cell context-dependent.

1. Introduction

The Ishikawa endometrial cancer cell line is derived from a well-differentiated endometrial adenocarcinoma [1] and these cells have been extensively utilized as an in vitro model to study endometrial carcinogenesis. Normal endometrium and Ishikawa cells express both the estrogen receptor (ER) and progesterone receptor (PR) [[2,3]]. 17β-Estradiol (E2) induces proliferation of Ishikawa cells [4–8]] and expression of genes and/or their dependent activities including alkaline phosphatase activity [5,9,10], PR levels [2,11], IGF-I mRNA levels [12], IGF-I receptor binding [13], TGFα mRNA and protein levels have been reported [8,14]. Many of these same responses are induced by E2 in other hormone-responsive tissues/cells including MCF-7 human breast cancer cells. For most of these responses, tamoxifen or 4′-hydroxytamoxifen exhibit ER agonist activity in Ishikawa cells and in uterine/endometrial tissues, this is consistent with the estrogenic effects of these compounds in endometrial/uterine tissues in laboratory animal studies [15]. Estrogenic activity of tamoxifen in the endometrial tissue is reason for concern due to the potential increased risk for endometrial cancer in women undergoing long term tamoxifen chemotherapy for breast cancer [16,17].

Aryl hydrocarbon receptor (AhR) agonists, typified by the environmental toxicant, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), have been extensively investigated as antiestrogens in the rodent mammary and uterus and human breast cancer cell lines [18]. For example, TCDD inhibited spontaneous and carcinogen-induced mammary cancer in female Sprague-Dawley rats and E2-induced mammary tumor growth in

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athymic B6C3F1 mice bearing MCF-7 cell xenografts [18–21]. TCDD also inhibits a diverse spectrum of E2-induced responses in MCF-7 cells including proliferation, DNA synthesis, PR binding, secretion of pS2, cathepsin D and tissue plasminogen activator activity, and pS2, cathepsin D and PR gene expression. TCDD also exhibits estrogenic activity in the rodent uterus and inhibits E2-induced uterine wet weight, peroxidase activity, PR and ER binding, EGF receptor and *fos* gene expression (reviewed in [18,22]). TCDD and related AhR agonists do not bind the ER and the antiestrogenic effects may be complex and are related to crosstalk between the AhR and ER signaling pathways [18,22].

Alternate substituted (1,3,6,8- or 2,4,6,8-)alkyl polychlorinated dibenzofurans (PCDFs), typified by 6-methyl-1,3,8-trichlorodibenzofuran (MCDF) have been developed as relatively nontoxic AhR-based antiestrogens for potential applications in the clinical treatment of breast cancer [23]. Like TCDD, MCDF and related compounds exhibit both antiestrogenic and antitumorigenic activity in the rodent mammary/uterus and human breast cancer cells [24,25]. This study investigates the Ah-responsiveness of Ishikawa endometrial cancer cells and shows that the AhR and Arnt are expressed, and MCDF, benzo[a]pyrene (BaP) and TCDD exhibit antiestrogenic activity. In contrast, ER/PR transcriptional interference with Ah-responsiveness was observed in ECC-1 endometrial cells, but not in Ishikawa cells indicating the importance of cell context for these interactions.

2. Materials and methods

2.1. Chemicals and biochemicals

TCDD, MCDF and [^{3}H]TCDD (30.0 Ci/mmol) was prepared in this laboratory and was determined to be > 99% pure by gas chromatography and spectroscopy. [^{3}H]17β-estradiol (122.5 Ci/mmol), [^{3}H]R5020 (86.7 Ci/mmol), and [^{14}C]chloramphenicol were purchased from NEN Research Products (Boston, MA). All other chemicals and biochemicals were of the highest quality available from commercial suppliers. The human wild type doubled-stranded CYP1A1 DRE oligonucleotide 5′-GATCTCCGGTCCTCACGCAACGCCTGGGG-3′ and mutant DRE 5′-GATCTCCGGTCCTTCTACAT-CAACGCCTGGGG-3′ were synthesized by Gene-Technologies Laboratory (College Station, TX). MCF-7, T47D and HeLa cells lines were purchased from American Type Culture Collection (Rockville, MD). Wild-type Hepa 1c1c7 cells were kindly provided by Dr. J.P. Whitlock, Jr. (Stanford University). Ishikawa human endometrial adenocarcinoma cells were a kind gift of Dr. Gurpide (Chester, CT).

2.2. Cell maintenance

Cells were maintained in DMEM-Ham's F-12 media (Sigma) with phenol red and supplemented with 2.2 g/l Na_2HCO_3, 5% fetal bovine serum (Intergen, Newark, NJ), and 10 ml/l antibiotic antimycotic solution (Sigma), pH 7.4. Cells were grown in 150-cm^2 culture flasks in an air:carbon dioxide (95%:5%) atmosphere at 37°C. Cells were passaged by trypsinization (0.1%).

2.3. Vectors and antibodies

The human estrogen receptor expression vector hER was kindly provided by Dr. Ming-Jer Tsai (Baylor College of Medicine, Houston, TX). The pRNH11c construct which contains the human CYP1A1 regulatory region (−1142 to +2434) fused to the chloramphenicol acetyltransferase (CAT) reporter gene was kindly provided by Dr. R.N. Hines (Wayne State University, Detroit, MI). The pPR-CAT construct containing the regulatory region (−2762 to +788) of the rabbit progesterone receptor gene was a kind gift of Dr. E. Milgrom (INSERM, France). The ERE-tk-CAT hybrid promoter construct that contains the estrogen responsive element (−331/−297) from the *Xenopus laevis* vitellogenin A2 gene fused to the thymidine kinase promoter was kindly provided by Drs. G.U. Ryffel and L. Klein-Hitpass (University Clinic, Essen, Germany). The pcDNA3.1/His/LacZ β-galactosidase transfection control plasmid was purchased from Invitrogen, Inc. (Carlsbad, CA). ER monoclonal antibodies C-314 and C-311 and anti-mouse secondary antibodies were purchased from Santa Cruz, Inc. (Santa Cruz, CA). The AhR polyclonal antibody was kindly provided by Dr. G. Perdew (Penn State University, University Park, PA), and the Arnt polyclonal antibody was a kind gift from Drs. Carol Holtzapple and Larry Stanker (Agricultural Research Service, USDA, College Station, TX).

2.4. Preparation of nuclear extracts

Cells were harvested by trypsinization and resuspended in HED hypotonic buffer (25 mM HEPES, 1.5 mM EDTA, 1 mM dithiothreitol, pH 7.6) and allowed to swell on ice for 10 min (all subsequent steps were performed on ice). Cells were then pelleted at 1000 × *g* for 5 min and homogenized in 0.5 ml HEGD buffer (HED plus 10% glycerol, pH 7.6) using a pestle drill apparatus resulting in > 90% membrane disruption determined by trypan blue staining. The homogenate was then centrifuged at 4000 × *g* for 10 min. The supernatant fraction was immediately centrifuged at

40,000 × *g* for 30 min to obtain cytosolic extract. The pellet containing nuclei and cellular debris was resuspended in an equal volume of HEGD buffer containing 0.5 M potassium chloride, incubated for 1 h on ice, then centrifuged at 40,000 × *g* for 30 min to obtain high salt nuclear extract.

2.5. Determination of nuclear Ah, ER and PR receptor levels

Forty-eight hours prior to dosing, cells were switched into DME/F-12 media without phenol red containing 5% charcoal stripped fetal bovine serum. Cells were then harvested by trypsinization, washed, resuspended in DME/F-12 (stripped) and treated with 10 nM [^{3}H]TCDD, [^{3}H]17β-estradiol, or [^{3}H]R5020 (promegestone). Baselines for AhR and ER were obtained by co-treatment with a 200-fold molar excess of unlabeled TCDD and diethylstilbesterol (DES), respectively. A PR baseline was established by co-treatment with a 500-fold molar excess of unlabeled progesterone. Following a 2 h incubation at 37°C, cells were centrifuged at 500 × *g*, washed twice in ice cold HEGD buffer, and nuclear extracts were obtained as described above. PR nuclear extracts were incubated with a 1:1 ratio of dextran-coated charcoal (DCC) for 15 min on ice to remove unbound [^{3}H]R5020 and DCC was removed by centrifugation at 4000 × *g* for 10 min at 4°C. Aliquots (200 μg ER and PR; 300 μg AhR) of radiolabeled nuclear extracts were layered onto linear sucrose gradients (5–25%) prepared in TEG buffer (0.25 mM Tris, 1.5 mM EDTA, 10% glycerol) containing 0.4 M potassium chloride. Following centrifugation at 404,000 × *g* for 2.5 h, gradients were fractionated into 30 equal fractions, and the radioactivity in each fraction was determined by liquid scintillation counting. Specific binding (fmoles receptor/mg protein) was calculated by subtracting the total radioactivity under the peak from the baseline (nonspecific binding) and using the specific activity of the radiolabeled ligands. Sedimentation coefficients (S) were calculated using a [^{14}C]BSA (bovine serum albumin) standard: $S^{\text{receptor}} = 4.4S^{\text{BSA}} \times$ receptor peak fraction number/BSA peak fraction number.

2.6. Electrophoretic mobility shift assays

Cells were treated with 10 nM TCDD or DMSO vehicle for 30 min. Cells were harvested by trypsinization, pelleted at 1000 × *g*, and nuclear extracts were obtained as described above. Nuclear protein (10 μg) and poly(dI-dC) (1 μg) were incubated for 15 min at 25°C. Following addition of 1 nM [^{32}P]-labeled specific DRE probe, the mixture was incubated for 15 min at 25°C. For competition with specific unlabeled or mutant DRE probe a 100-fold excess was incubated for 15 min prior to the addition of [^{32}P]-labeled DRE. The reaction was carried out in 25 mM HEPES, 1.5 mM EDTA, 10% glycerol 1 mM dithiothreitol (HEGD) in a final volume of 30 μl. Reaction mixtures were loaded onto a 5% polyacrylamide gel and run at 120 V in 90 mM Tris, 90 mM borate, 2 mM EDTA (pH 8.0). Gels were dried and protein–DNA binding was visualized by autoradiography.

2.7. Ethoxyresorufin O-deethylase (EROD) activity

Cells were seeded into 48-well plates in 0.2 ml of maintenance media. After 24 h, plates were treated with 0.1, 1.0, or 10 nM TCDD or DMSO vehicle. The kinetic conversion of ethoxyresorufin to resorufin was used as an indicator of CYP1A1-dependent activity. EROD activity and protein determinations were measured in the same samples as described by Kennedy and Jones [[26]]. Analysis was conducted on a CytoFluor 2350 plate reader at 530 nm/590 nm for resorufin production and 400 nm/460 nm for fluorescamine protein determination.

2.8. Cell proliferation assay

Ishikawa cells were seeded into 35 mm 6-well tissue culture plates at a density of 50,000 cells/well in DME/F12 maintenance media. The following day maintenance media was replaced with phenol-free, serum-free DME/F12 media supplemented with sodium bicarbonate (2.2 g/l), 10 ml/l antibiotic–antimycotic solution, insulin (6.25 μg/ml), transferrin (6.25 μg/ml), selenium (6.25 ng/ml), bovine serum albumin (1.25 mg/ml) and linoleic acid (5.35 μg/ml). Cells were then treated with test compounds for 8–12 days; the media was changed and cells were re-dosed with compounds every two days. Cells were then harvested by trypsinization and counted using a Coulter cell counter.

2.9. Plasmid transfection studies

Cultured Ishikawa cells were transiently transfected for 18 h by calcium phosphate coprecipitation with 10 μg of reporter plasmids and 5 μg of pcDNA3.1/His/LacZβ-galactosidase control vector. The pPR-CAT reporter plasmid was cotransfected with 5 μg of hER expression vector. pRNH11c was transfected and treated in maintenance media. All other reporter plasmids were transfected and treated in phenol-free DMEM-Ham's F-12 with 5% stripped fetal bovine serum. Following transfection cells were treated with compounds in fresh media for 24 h. Cells were harvested, rinsed with phosphate-buffered saline, scraped from the plate, and centrifuged for 5 min at 12,000 rpm in a Eppendorf centrifuge. The resulting pellet was resuspended in 0.1 ml of 0.25 M Tris–HCl (pH 7.5), and lysates were

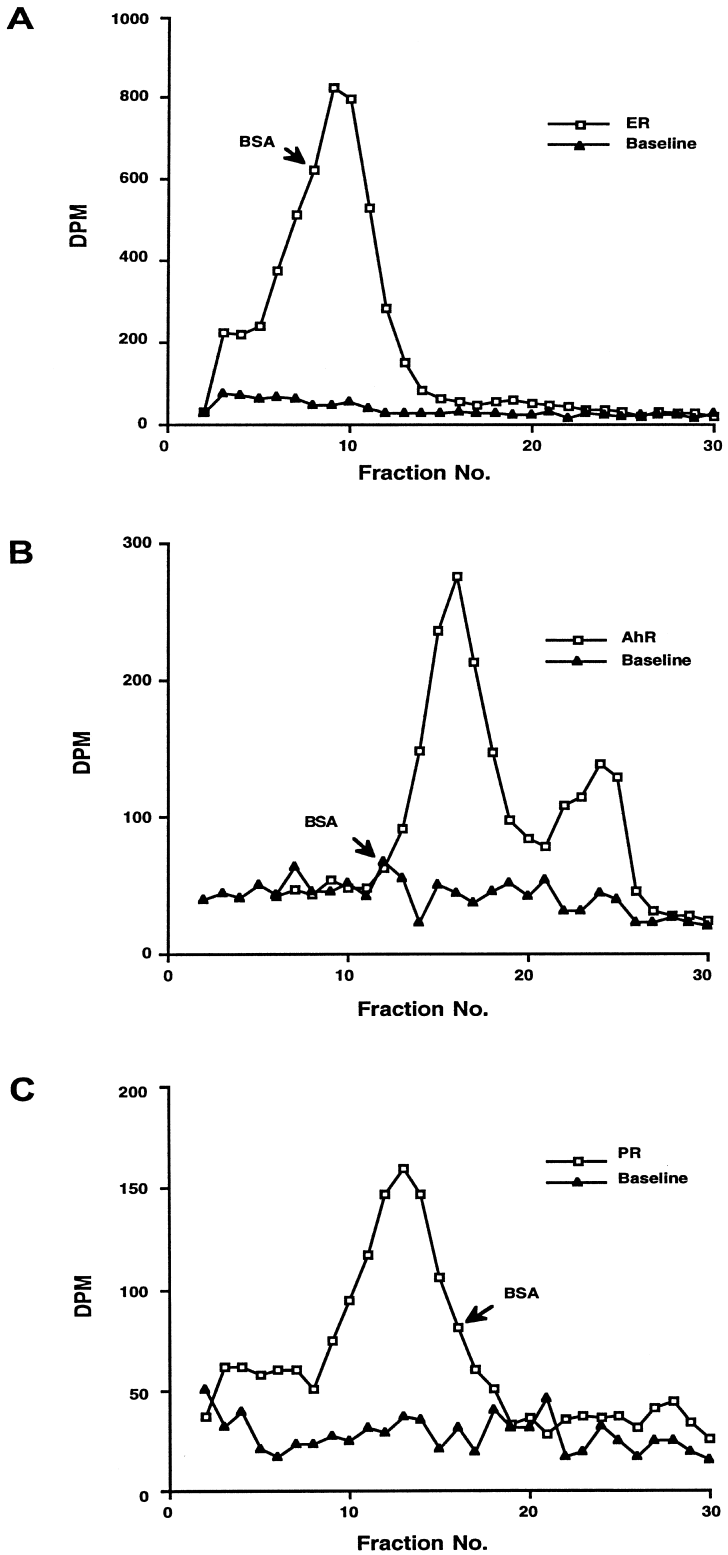

Fig. 1. Velocity sedimentation analysis of nuclear extracts from Ishikawa cells. Nuclear extracts were obtained and analyzed by sucrose density gradient centrifugation as described in Section 2. Ishikawa cells were treated with 10 nM [^{3}H]E2 (A), 10 nM [^{3}H]TCDD (B), or 10 nM [^{3}H]R5020 (C). Levels of specifically-bound radiolabeled nuclear ER, AhR, and PR were 72 ± 1.1, 67 ± 1.4, and 19.3 ± 0.8 fmol/mg, respectively. Sedimentation coefficients for ER, AhR, and PR were 5.0 S, 6.0 S, and 3.5 S, respectively. Arrow indicates peak fraction number where 4.4 S bovine serum albumin (BSA) spiked standard eluted. Baselines were generated with non-radiolabeled cotreatments of 200× diethylstilbestrol (A), 200× TCDD (B), and 500× progesterone (C).

prepared by three cycles of freeze–thawing followed by centrifugation for 5 min at 12,000 rpm to obtain supernatant. Equal amounts of protein from each sample were diluted in 0.25 M Tris–HCl (pH 7.5) and incubated at 56°C for 5 min to remove endogenous deacetylase activity. D-Threo-[dichloroacetyl-1-^{14}C]chloramphenicol (0.2 mCi, NEN Life Science Products) and 4 mM acetyl-CoA were added and incubated for 18 h in a 37°C water bath. After incubation 700 μl of ethyl acetate was added, and samples were vortexed and centrifuged for 5 min at 12,000 rpm. A 600 μl aliquot of the upper phase was transferred to a fresh Eppendorf tube, dried, and dissolved in 20 μl of ethyl acetate, and spotted onto a Whatman PE SilG thin layer chromatography (TLC) plate (Kent, UK), and the substrate and acetylated products were resolved using chloroform:methanol (95:5%). Acetylated products were visualized and quantitated using a Packard Instant Imager (Packard Instrument, Meridian, CT). All CAT assays were normalized for β-galactosidase activity and calculated as a fraction of that observed in cells treated with DMSO alone.

2.10. Statistical analysis

Results are presented as means ± SE for at least three determinations for each treatment group. All experiments were carried out at least two or more times. Statistical differences between groups were determined by analysis of variance followed by Fischer's protected LSD test for significance (SuperANOVA, Abacus Concepts, Berkeley, CA) using $p \leq 0.05$ as the level of significance.

3. Results

3.1. ER and PR expression in Ishikawa cells

Ishikawa cells were treated with 10 nM [^{3}H]E2 for 2 h; nuclear extracts were isolated and analyzed by sucrose density gradients to give a 5.0 S specifically-bound radiolabeled complex (Fig. 1C). Estimated nuclear ER levels were 72 fmol/mg protein. Using a similar protocol and [^{3}H]R5020, a 3.5 S specifically-bound PR complex was also observed (Fig. 1A) and PR levels were approximately 19 fmol/mg protein. In parallel studies with MCF-7 human breast cancer cells, the specifically-bound ER and PR complexes were indistinguishable from those isolated from Ishikawa cells (data not shown). Gel mobility shift assays using [^{32}P]ERE and nuclear extracts from Ishikawa and MCF-7 cells showed that a specifically-bound ER-ERE retarded band was also formed with extracts from both cell lines (data not shown). Immunoreactive ERα(66-kD) protein was also detected byWestern blot

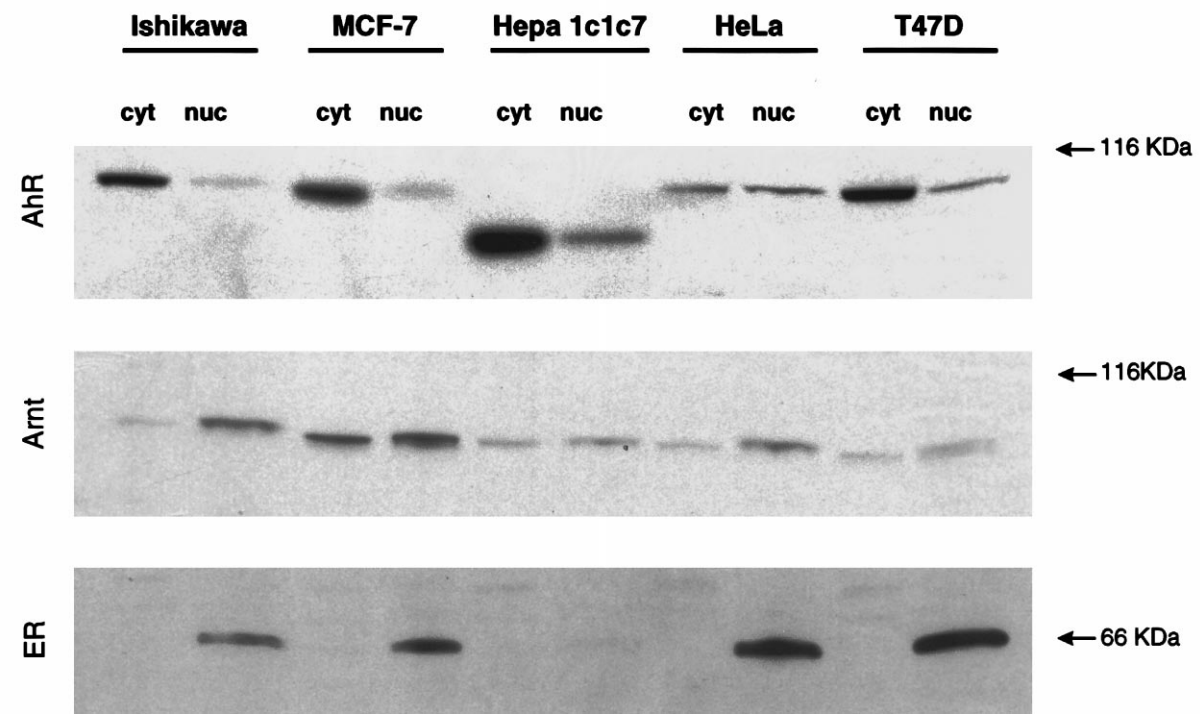

Fig. 2. Western blot analysis of cytosolic and nuclear AhR (A), Arnt (B), and ER (C) proteins in Ishikawa cells and other cancer control cell lines. Cells were incubated in maintenance media and extracts were prepared as described in Section 2. Cytosolic and nuclear protein (125 μg) from Ishikawa, MCF-7, Hepa 1c1c7, HeLa and T47D cells were separated by SDS-PAGE (7.5%) and transferred to PVDF membrane for immunoblotting and autoradiography.

analysis in Ishikawa, MCF-7, T47D and HeLa cells only in the nuclear fraction, whereas the protein was nondetectable in mouse Hepa 1c1c7 liver cancer cells (Fig. 2C).

3.2. Comparative AhR and Arnt expression in Ishikawa and other cancer cell lines and induction of EROD activity

Sucrose density gradient analysis of nuclear extracts from cells treated with 10 nM [^{3}H]TCDD for 2 h gave a 6.0 S specifically-bound peak and estimated levels of the nuclear AhR complex were 45 fmol/mg protein (Fig. 1B). The results in Fig. 2A and B show that immunoreactive AhR and Arnt are primarily expressed in cytosolic and nuclear fractions, respectively, in Ishikawa cells. Distribution of the AhR in ER-positive MCF-7 and T47D cells was similar to Ishikawa cells, whereas in HeLa cells, comparable AhR levels were detected in both cytocolic and nuclear fractions. Immunoreactive Arnt protein was detected in both cytosolic and nuclear fractions. A comparison of AhR complex binding to [^{32}P]DRE was also determined in Ishikawa and MCF-7 cells using nuclear extracts from cells treated with DMSO (D) or TCDD (T) (Fig. 3A). In both cell lines, 10 nM TCDD induced a specifically bound retarded band (DRE→) that was competitively decreased by 100-fold excess unlabeled wild-type but not mutant DRE. The comparative effects of DMSO vs. TCDD treatment on cytosolic and nuclear immunoreactive AhR and Arnt proteins were also determined; TCDD enhanced formation of nuclear AhR and Arnt proteins in Ishikawa cells (Fig. 3B), and these results are consistent with the gel mobility shift data (Fig. 3A).

Treatment of Ishikawa cells with TCDD caused a concentration-dependent induction of EROD activity, and a maximum 9.7-fold induction response was observed using 10 nM TCDD (Fig. 4). Similar results were obtained in transient transfection studies using pRNH11c, an Ah-responsive construct containing the −1142 to +2434 region of the human CYP1A1 gene

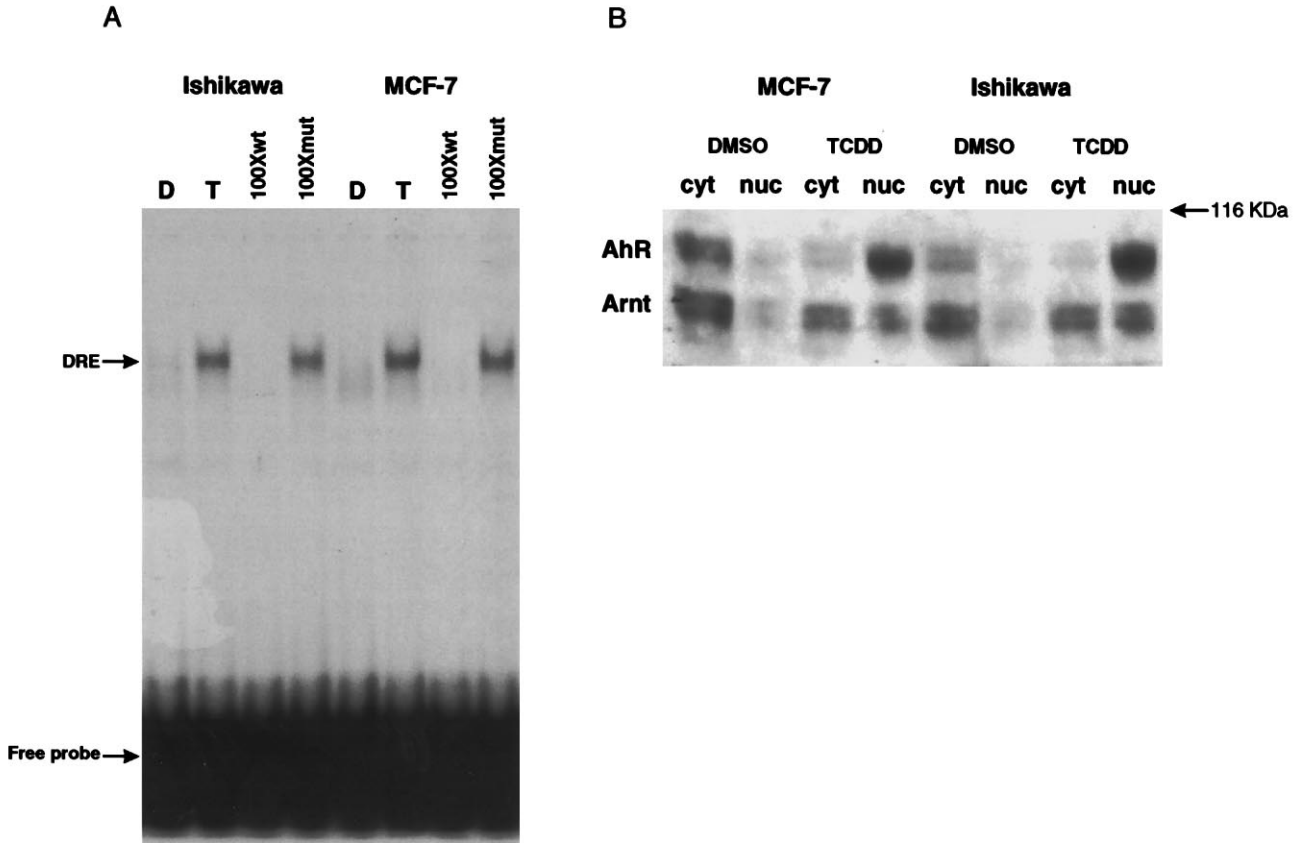

Fig. 3. Binding of nuclear extracts from Ishikawa and MCF-7 cells to dioxin response element (DRE), and nuclear translocation of AhR and Arnt proteins. (A) Nuclear extracts were analyzed by electromobility shift assay (EMSA). Cells were treated with DMSO vehicle (D) or 10 nM TCDD (T) for 30 min prior to harvesting. The retarded [^{32}P]DRE bands (see arrow) were visualized by autoradiography. TCDD-treated extracts competed with 100× non-radiolableled wild-type (100× wt) or mutant (100× mut) DRE probe confirmed specificity. (B) Cytosolic and nuclear extracts (100 μg) from Ishikawa and control MCF-7 cells treated with DMSO or 10 nM TCDD for 30 min were separated by SDS-PAGE (10%) and transferred to PVDF membrane for immunoblotting and autoradiography. TCDD-induced translocation of AhR and Arnt proteins to the nuclear fraction is evident in both cell lines. Cells were incubated and dosed in maintenance media.

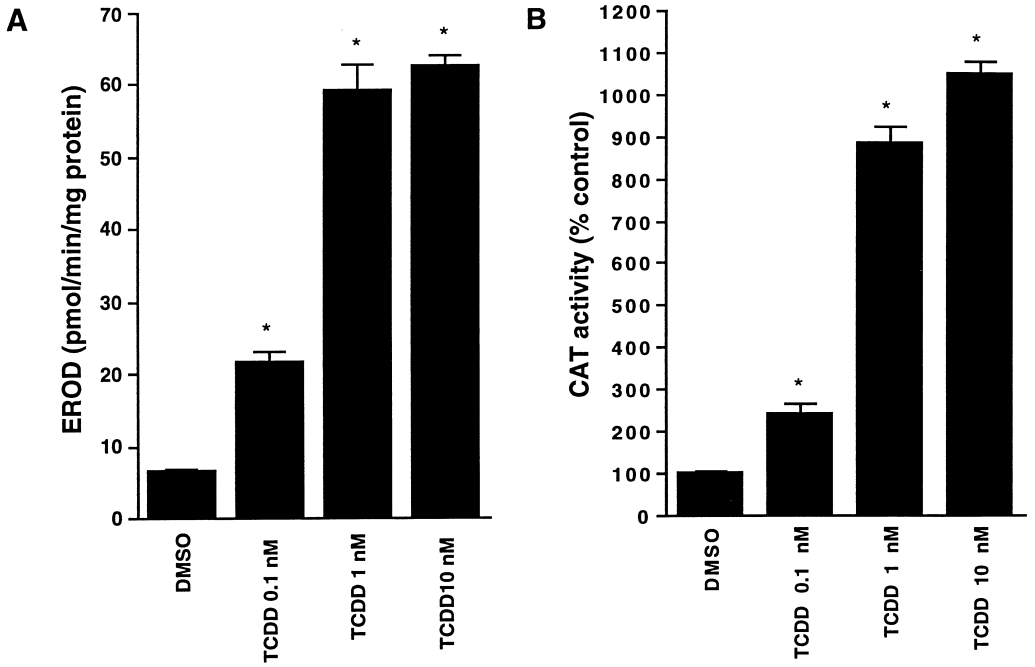

Fig. 4. Induction of EROD and CAT activity in Ishikawa cells. (A) Ishikawa cells were treated with DMSO (vehicle) or 0.1, 1, and 10 nM TCDD for 24 h, and EROD activity was measured as described in Section 2. (B) Ishikawa cells were transiently transfected with the pRNH11c CAT reporter construct containing the 5′-regulatory region of the CYP1A1 promoter, and were then treated as described above for 24 h. Cells were harvested and CAT activity was measured as described in Section 2. Data are expressed means ± SE ($n = 3$). [a]Significantly higher ($p < 0.05$) than observed for controls (DMSO) group.

promoter.Ten nM TCDD caused a >10-fold increase in CAT activity (Fig. 4A). Thus, Ishikawa cells express functional AhR and Arnt proteins.

3.3. Inhibitory AhR–ER crosstalk

Previous studies have demonstrated that AhR agonists inhibit E2-induced proliferation and gene expression in ER-positive breast cancer cells [18,22], and the growth inhibitory effects of TCDD, BaP and MCDF (a relatively nontoxic AhR agonist) were determined in Ishikawa cells (Fig. 5). TCDD, BaP and MCDF alone did not affect proliferation of Ishikawa cells (data not shown), whereas 1 or 10 nM E2 induced a 2.2- to 4.7-fold increase in cell proliferation (Fig. 5A). In cells cotreated with E2 plus the AhR agonists TCDD, BaP and MCDF, there was a 35–47% decrease in E2-induced cell growth. Inhibition of E2-induced transactivation by AhR agonists was determined in Ishikawa cells transiently transfected with two E2-responsive constructs, pPR and pERE, containing −2762/+788 and −331/−297 promoter insert from the rabbit progesterone receptor and *Xenopus laevis* vitellogenin A2 gene promoters, respectively [27,28]. The results show that both TCDD and BaP inhibit E2-induced CAT activity using both promoters (32–39% range of inhibition) (Fig. 5B–D).

3.4. Role of hormones on Ah-responsiveness

Some studies have reported that steroid hormones decrease CYP1A1-dependent activity in E2-responsive breast and endometrial cancer cell lines, whereas these interactions have not been observed in other reports [29–33]. ECC-1 endometrial cancer cells are both Ah- and E2-responsive [33,34], and therefore, the role of steroid hormones on Ah-responsiveness was investigated in both cell lines (Fig. 6). The result show that E2 significantly inhibited induction off EROD activity by TCDD in ECC-1 cells, and this inhibitory response was reversed by cotreatment of ECC-1 cells with E2 plus 4′-hydroxytamoxifen. In contrast, progesterone did not affect induction of EROD activity by TCDD in ECC-1 cells (Fig. 6A). In parallel experiments with Ishikawa cells, neither E2, tamoxifen, or progesterone modulated induction of EROD activity by TCDD (Fig. 6B), demonstrating that hormone-mediated modulation of Ah-responsiveness is cell specific.

4. Discussion

Ishikawa endometrial cancer cells were initially identified as an E2-responsive cell line that can be used as a model for understanding hormonal and growth factor-mediated cell proliferation and gene expression

[1–14]. Some studies have demonstrated variable responses of Ishikawa cells to mitogenic agents and this was due, in part, to growth media and may also be related to growth- and passage-dependent genotypic cell variability [4–8]. For example, some Ishikawa cells express low ER_α levels and exhibit minimal E2-responsiveness [35,36]. Therefore, Ishikawa cells utilized in these studies were initially characterized for their E2-responsiveness. These cells expressed both ER_α and PR (Figs. 1 and 2); E2 induced cell proliferation and transactivation in transient transfection assays (Figs. 3 and 4) and bound a [^{32}P]ERE in gel mobility shift assays (data not shown). The mitogenic effects of E2 and 4′-hydroxytamoxifen were similar to those previously reported and were only observed in serum-free culture condition [4–8]; in the presence of charcoal-

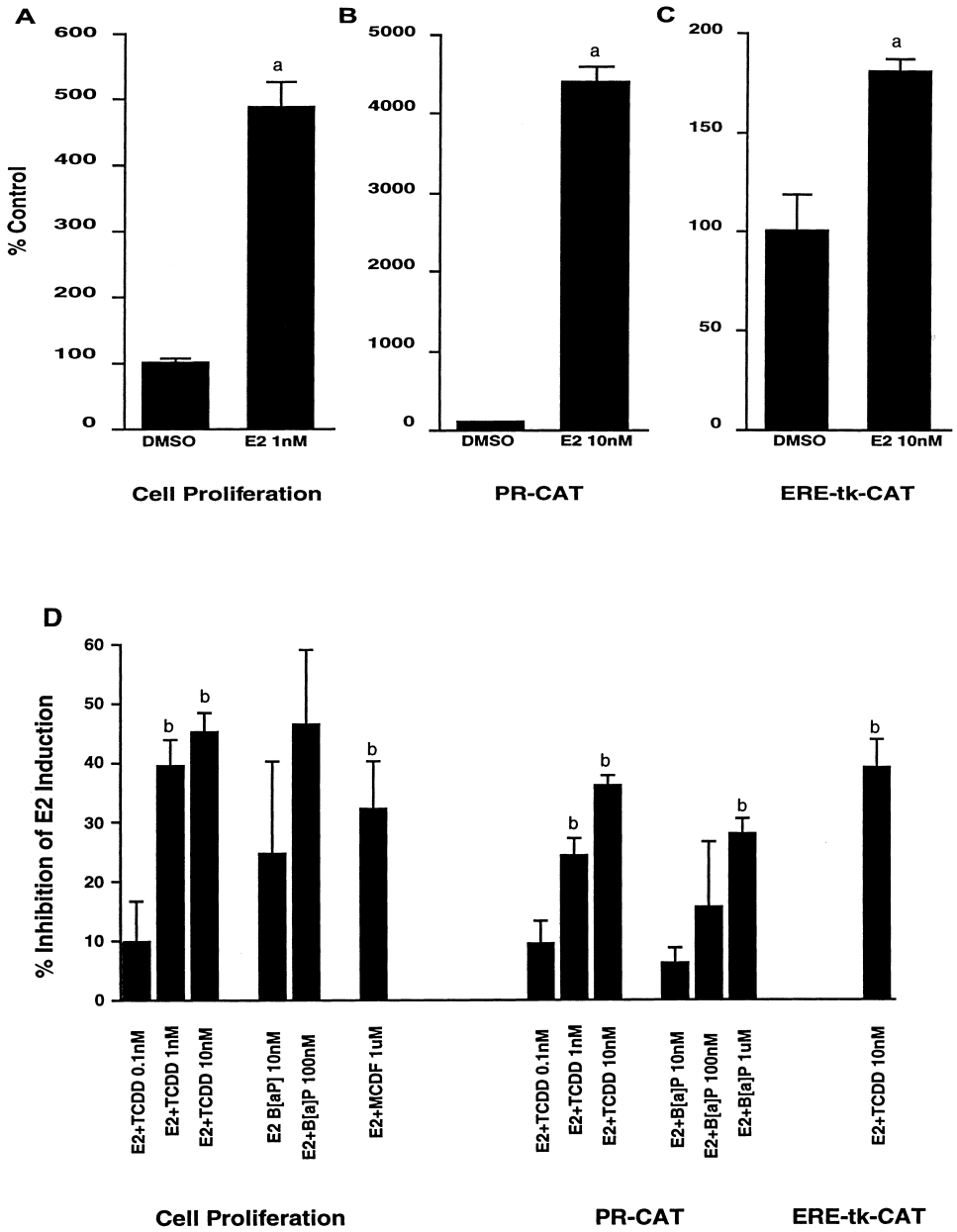

Fig. 5. Effects of E2, TCDD, BaP and MCDF on cell proliferation and estrogen-responsive CAT activity. Cell proliferation (A and D): Ishikawa cells were grown for 8–11 days in supplemented serum-free media as described under Materials and Methods. The number of cells in each treatment group was determined using a Z1 Coulter Counter. Reporter gene activity (B and D): The CAT reporter construct containing the 5′-regulatory sequence from the rabbit progesterone receptor gene (PR-CAT) or consensus ERE from *Xenopus laevis* vitellogenin gene (ERE-tk-CAT) was transiently cotransfected with the human estrogen receptor (hER) expression vector. CAT activity was determined as described in Section 2. At least three replicates were used for each treatment group and the results are expressed as means ± SE. BaP, TCDD and MCDF alone did not affect cell proliferation or CAT activity (data not shown). [a]Significantly higher ($p < 0.05$) than observed for DMSO-treated groups. [b]Significantly lower ($p < 0.05$) than observed for E2-treated groups.

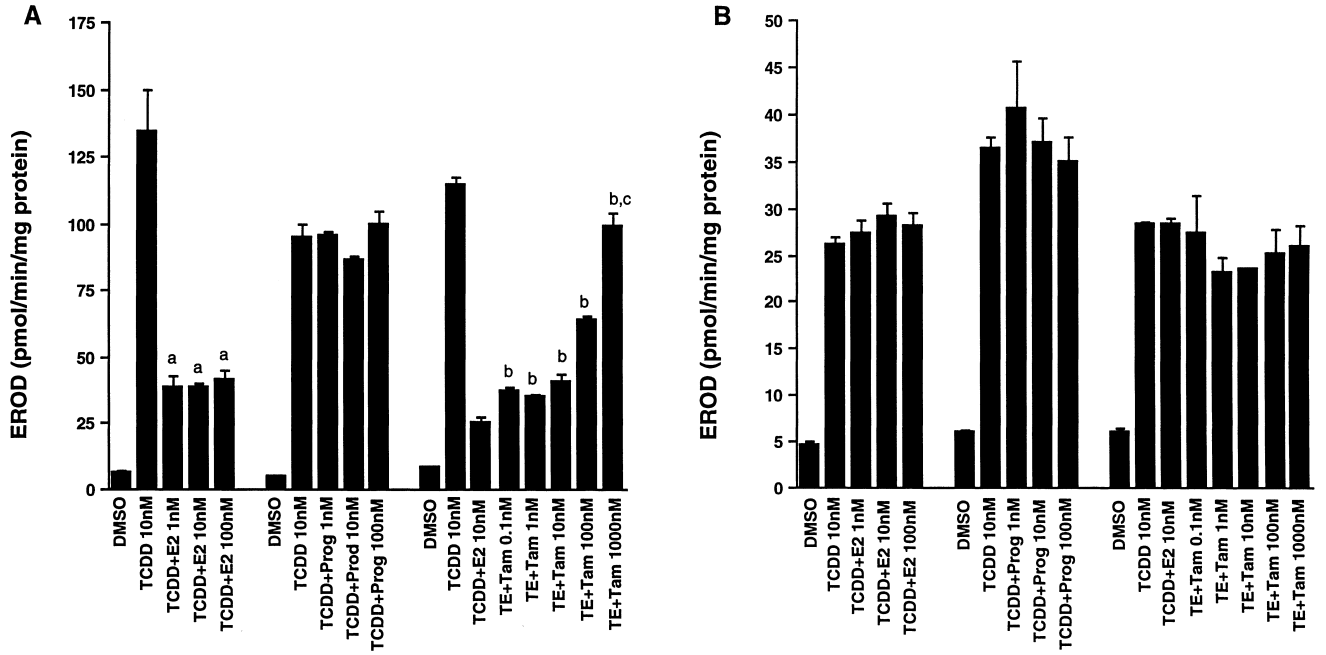

Fig. 6. Effects of estrogen and progesterone on induction of EROD activity in Ishikawa and ECC-1 cells. ECC-1 (A) and Ishikawa (B) cells were treated with DMSO (vehicle), 10 nM TCDD (T) alone, or in combination with E2 (E), progesterone (Prog) or 4′-hydroxytamoxifen (Tam). EROD activity was measured as described in Section 2. E2 significantly inhibited TCDD-induced EROD activity in ECC-1 cells, and this inhibition was reversed by increasing concentrations of 4′-hydroxytamoxifen (A). E2 had no significant effect on induced EROD activity in Ishikawa cells (B). EROD activity was not significantly altered by cotreatment with progesterone in either cell line. Data are expressed as means ± SE ($n = 3$). [a]Significantly lower ($p < 0.05$) than observed for TCDD alone. [b]Significantly lower ($p < 0.05$) than observed for TCDD + E2. [c]Not significantly different from TCDD alone.

stripped serum neither E2 or 4′-hydroxytamoxifen-induced proliferation of Ishikawa cells (data not shown).

Research in this laboratory has focused on the mechanisms of AhR-mediated inhibition of estrogenic action (reviewed in [18,22]) and development of AhR-based antiestrogens for treatment of both E2-responsive breast and endometrial cancers [18,22–25,34,37]. Previous studies have demonstrated that TCDD, a potent AhR agonist, inhibited E2-induced growth and gene expression in human breast cancer cells and rodent mammary tumor models [19–21] and also blocked rodent uterine responses induced by E2 [18,22]. Results of this study demonstrate that the AhR and Arnt proteins are expressed in Ishikawa cells and bind to [^{32}P]DRE in a gel mobility shift assay (Figs. 1–3). Ah-responsiveness was confirmed by induction of CYP1A1-dependent EROD and reporter gene activity (using pRNH11c) by TCDD (Fig. 4), and similar effects have previously been reported for ER-positive MCF-7 and T47D breast cancer cell lines [38].

Crosstalk between Ah receptor and ER signaling pathways in Ishikawa cells was investigated using cell proliferation and transactivation assays (Fig. 5). TCDD, BaP and MCDF, a relatively nontoxic AhR agonist, inhibited E2-induced cell growth and reporter gene activity in Ishikawa cells transiently transfected with E2-responsive pERE and pPR constructs. It is also noteworthy that the antiestrogenic activity of the prototypical polynuclear aromatic hydrocarbon (PAH) combustion by-product, BaP, in Ishikawa cells is consistent with human in vivo data showing that smoking in women is associated with a decreased incidence of hormone-dependent endometrial cancer [39]. Inhibitory responses by the different structural classes of Ah receptor agonists were approximately 25–40% and the magnitude of these effects were lower than observed for pure antiestrogens such as ICI 182,780 or ICI 164,384 [40]. The modest antiestrogenic responses for AhR agonists observed in Ishikawa cells contrasted to the < 50–100% antiestrogenic potencies of the same compounds in ECC-1 endometrial cancer cells and these inhibitory responses were comparable to those observed for "pure" antiestrogens [34]. Differences between cell lines may be related, in part, to the higher E2-responsiveness of ECC-1 cells compared to Ishikawa cells; moreover, in the former cell line 4′-hydroxytamoxifen is primarily an ER antagonist [34] compared to the ER agonist activity of this compound in Ishikawa cells [2,4,8].

Recent studies have reported seemingly contradictory effects of E2 and progesterone on Ah-responsiveness in E2-responsive cells [29–33]. For example, E2 decreased TCDD-induced CAT activity in MCF-7

cells transfected with a DRE-dependent construct in one study [29]; in contrast, this inhibitory response was not observed by Hoivik and co-workers [30], and these differences may be related, in part, to highly variable ER expression in MCF-7 cells from various laboratories [41] Transcriptional interference of AhR-dependent transactivation by progestins was reported in both T47D breast and HepG2 liver cancer cell lines (cotransfected with PR expression plasmids) [32]. Ricci and co-workers [31,33] also reported ligand-activated ER interference with induction of CYP1A1 by TCDD in ECC-1 cells; moreover, they showed that cotransfection with nuclear factor 1 significantly reversed the inhibitory effect of ER. Results summarized in Fig. 6 confirm that treatment of ECC-1 cells with E2 also significantly decreased induction of CYP1A1-dependent EROD activity by TCDD, whereas progesterone did not affect the induction response (Fig. 6A). In contrast, neither E2 or progesterone significantly affected Ah-responsiveness of Ishikawa cells (Fig. 6B) suggesting that ER and PR interference with AhR-mediated transactivation is dependent on cell context. Thus, transcriptional interference between ER/PR and Ah-responsiveness is probably dependent on cell type-specific expression of various transcription factors including nuclear receptor coactivators that functionally interact with both ER/PR [42] and the AhR complex [43]. Moreover, these interactions may vary within the same cell line since both hormone- and Ah-responsiveness can vary with growth conditions and cell passage.

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