Estrogen Does Not Inhibit 2,3,7,8-Tetrachlorodibenzo-p-dioxin-mediated Effects in MCF-7 and Hepa 1c1c7 Cells*

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The estrogen receptor and aryl hydrocarbon receptor (AhR) are coexpressed in several Ah and estrogen-responsive human breast cancer cell lines. However, a recent study reported that 17β-estradiol (E2) inhibited Ah responsiveness in mouse Hepa 1c1c7 hepatoma cells (Kharat, L., and Saatcioglu, F. (1996) J. Biol. Chem. 271, 10533–10537), and therefore, estrogen receptor-AhR cross-talk was reinvestigated in MCF-7 and mouse Hepa 1c1c7 cells. Treatment of MCF-7 or Hepa 1c1c7 cells with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) resulted in induction of CYP1A1-dependent activity and mRNA levels. Treatment of both cell lines with E2 had no effect on basal or TCDD-inducible CYP1A1-dependent activity or mRNA levels. In MCF-7 and Hepa 1c1c7 cells transiently transfected with an Ah-responsive plasmid containing the 5′-regulatory region of the human CYP1A1 gene fused to the chloramphenicol acetyltransferase reporter gene 10 nm TCDD significantly induced chloramphenicol acetyltransferase activity; in cells cotreated with TCDD plus E2 the induced response was not affected by the hormone. Nuclear extracts from cells treated with dimethyl sulfoxide, E2, TCDD, and TCDD plus E2 were incubated with the [32P]dioxin-responsive element and analyzed by gel electrophoretic mobility shift assays. A retarded band associated with formation of a [32P]dioxin-responsive element-AhR complex was observed in nuclear extracts from cells treated with TCDD or TCDD plus E2 (cotreated). Collectively these studies suggest that E2 does not modulate AhR-mediated CYP1A1 gene expression in MCF-7 or Hepa 1c1c7 cells.

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is an environmental contaminant that elicits a number of toxic and biochemical responses including chloracne, carcinogenesis, thymic atrophy and immune suppression, hypo/hyperplasia, hepatoxicity, tumor promotion, reproductive and developmental toxicity, and induction of CYP isoenzymes and other drug-metabolizing enzymes (1). The aryl hydrocarbon receptor (AhR) has been identified as the initial cellular target for TCDD and related compounds, and results of most studies indicate that the effects of these chemicals are mediated through the AhR. The AhR is a ligand-induced nuclear transcription factor, which forms a heterodimer with the AhR nuclear translocator protein. Based on extensive studies with the CYP1A1 gene, ligand-induced transactivation is associated with interaction between the nuclear AhR complex and cis-genomic dioxin-responsive elements (DREs) located in the 5′-promoter region of responsive genes (2).

Several studies have shown that TCDD modulates estrogen (E2)-induced pathways in human breast cancer cells. For example, treatment of MCF-7 cells with E2 increases cell proliferation (3); prothrombin D, cathepsin D, and pS2 (5) secretion; progesterone receptor (6) and HSP27 (7) and c-fos (8) gene expression; in cells cotreated with TCDD plus E2 all of the hormone-induced responses were decreased. The mechanism of AhR-mediated inhibition of E2-induced cathepsin D (9) and HSP27 (7) and pS2 (5) gene expression involves interactions of the nuclear AhR complex with inhibitory dioxin-responsive elements strategically located in the promoter regions of these genes.

Vickers et al. (10) first pointed out that for a series of ER-positive and ER-negative breast cancer cell lines only the former cells were Ah-responsive as determined by induction of CYP1A1 gene expression by TCDD. In contrast, breast cancer cell lines such as ER-negative MDA-MB-231 (11) and HS578T (12) cells express the AhR and AhR nuclear translocator proteins, but TCDD did not induce CYP1A1 or reporter gene activity in cells transiently transfected with Ah-responsive constructs. Research in this laboratory showed that transient transfection of the ER or variant ERs expressing activation function 1 or activation function 2 restored Ah responsiveness in MDA-MB-231 and HS578T cells (11, 12). Although the mechanisms of cross-talk between the ER and AhR are unknown the results suggest that the ER plays an important role in AhR-mediated gene expression in both ER-positive and ER-negative human breast cancer cell lines. Surprisingly, Kharat and Saatcioglu (13) reported that E2 significantly inhibited TCDD-induced reporter gene activity in Hepa 1c1c7 cells transiently transfected with an Ah-responsive construct. Moreover, in gel mobility shift assays using nuclear extracts from cells treated with TCDD or TCDD plus E2 (cotreated), they reported that hormone treatment blocked formation of the TCDD-induced retarded band. These data were inconsistent with results of previous studies, and therefore the effects of E2 on Ah responsiveness have been reinvestigated in both MCF-7 and Hepa 1c1c7 cells. The results of this study clearly demonstrate that E2 does not affect Ah responsiveness in either cell line.

MATERIALS AND METHODS

Chemicals and Biochemicals—TCDD was prepared in this laboratory and was determined to be >99% pure by gas chromatography and mass spectroscopy. All other chemicals and biochemicals were of the highest quality available from commercial suppliers. Pure ER was purchased from PanVera (Madison, WI). The human wild type double-stranded DRE oligonucleotide 5′-GATCTCGGCTCTCATCAGCGA-

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1 The abbreviations used are: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; AhR, aryl hydrocarbon receptor; DRE, dioxin-responsive element; E2, estrogen; ER, estrogen receptor; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; EROD, ethoxyresorufin O-deethylase.
ACGCTGGG-3' and mutant DRE 5'-GATCTCCGCTCTCTA-3' were synthesized by Gene Technologies Laboratory (College Station, TX).

Cell Maintenance—The human adenocarcinoma (MCF-7) and mouse hepatoma (Hepa 1c1c7) cell line were obtained from the American Type Culture Collection (Rockville, MD). MCF-7 cells were grown in Dulbecco's modified Eagle's medium (Life Technologies, Inc.) supplemented with 10% fetal bovine serum (Intergen, Newark, NJ), 10 μg/liter insulin, 2.38 g/liter HEPES, 1.0 g/liter glucose, 2.2 g/liter NaHCO₃, 0.11 g/liter sodium pyruvate, and 2 ml/liter antibiotic/antimycotic (Sigma) solution. Hepa 1c1c7 cells were grown in Dulbecco's modified Eagle's/F12 medium supplemented with 5% fetal calf serum and 2.2 g/liter NaHCO₃ and 10 ml/liter antibiotic/antimycotic solution. All cells were grown on 150-cm² tissue culture dishes in a 37 °C incubator with a humidified mixture of 5% CO₂ and 95% air. Cells were subcultured at approximately 80% confluency. For induction experiments cells were grown in Dulbecco's modified Eagle's/F12 medium supplemented with 2.5% charcoal-stripped serum and 2.2 g/liter NaHCO₃ and 10 ml/liter antibiotic/antimycotic solution for 2 days prior to treatment and then maintained in this medium throughout the experiment.

Expression Vectors—The pRNH11c plasmid containing the −1140 to +2435 promoter region of the human CYP1A1 gene fused to the bacterial chloramphenicol acetyltransferase (CAT) reporter gene was kindly provided by D. Ronald Hines (Wayne State University).

Oligonucleotides—Oligonucleotides were synthesized by the Gene Technologies Laboratory, Institute for Developmental and Molecular Biology, Texas A&M University or by Genosys (The Woodlands, TX). Primers used to amplify human and mouse CYP1A1 were as follows: B0350, 5'-TAGACTAGTCTGCTGAGCAG-3'; B0351, 5'-GGGAAGGCTCTCAACAATGCT-3'; mCYP1A1F, 5'-ACACTACAGACGCTCCTAGTGA-3' and 5'-CCACTGGTTCACAAAGACACA-3', respectively. Optimal annealing temperature and cycle number for each primer set were determined empirically to be 60 °C (23 cycles) for B0350/B0351 and 58 °C (25 cycles) for mCYP1A1F/mCYP1A1R.

Competitive Reverse Transcriptase-Polymerase Chain Reaction—PCR reagents were purchased from Perkin-Elmer, and all experiments were performed using a Perkin-Elmer GeneAmp PCR System 9600 (Foster City, CA) or MJ Research DNA Engine (Watertown, MA) in a thin walled reaction tubes (Phenix, Hayward, CA). Total RNA was extracted by the RNAzol method (Tel-Test, Friendswood, TX) and quantitated by UV spectroscopy. Semiquantitative reverse transcriptase-PCR using exogenous internal controls was used for analysis of CYP1A1 messenger RNA in mouse and human cells. A 592-base pair internal control was produced by reverse transcribing 200 ng of total RNA from MCF-7 or Hepa 1c1c7 cells followed by 30 cycles of PCR using primers containing a sequence specific to human β-actin ligated distally to sequences containing primer sites for amplification of human or mouse CYP1A1 (see above). PCR products were preparatively separated on a 6% (w/v) polyacrylamide gel (National Diagnostics, Atlanta, GA), excised using a sterile scalpel blade, and extracted using a modified crush and soak method (14). The forward primer B0350F or mCYP1A1F was end-labeled with [α-32P]dATP (3000 Ci/mmol, 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 the samples were mixed by vortexing and centrifuged for 5 min in an Eppendorf centrifuge at 12,000 rpm. A 600-μl aliquot of the upper phase was transferred to a fresh Eppendorf tube, dried, and then dissolved in 20 μl of ethyl acetate, and spotted onto a Whatman PSiG thin layer chromatography (TLC) plate (Kent, UK), and the spots were visualized using chloroform/methanol (95:5%). The TLC plate was exposed to Kodak XAR-5 film overnight for visualization of radiolabeled acetylated metabolite.

Determination of Ethoxyresorufin O-Deethylase (EROD) Activity—Cells were seeded into 48-well plates at a density of 60,000 cells/well in 0.5 ml of media. After 24 h, plates were treated with 1 nM TCDD or Me₂SO 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 (16). Analysis was conducted on a CytoFluor 2350 plate reader at 530 nm/590 nm for resorufin production and 400 nm/460 nm for fluorescein protein determination.

Electrophoretic Mobility Shift Assays—Cells were treated with 10 nM TCDD either in the presence or absence of 10 nM E2 or Me₂SO vehicle for 1 h. Cells were harvested and washed with HEPES buffer (25 mM HEPES, 1.5 mM EDTA, 1 mM dithiothreitol, 10% glycerol (pH 7.6)) and centrifuged for 5 min at 800 × g (4 °C). The resulting pellet was resuspended in 1 ml of 0.25 M Tris-HCl (pH 7.5) and lysates were prepared by 3 min (times 3) of freeze-thaw and sonication. Cell lysates were centrifuged for 5 min at 12,000 rpm, the supernatant was decanted, and the protein concentration was determined using bovine

FIG. 1. Induction of EROD activity in MCF-7 and Hepa 1c1c7 cells. MCF-7 (A) and Hepa 1c1c7 (B) cells were treated with either 1 nM TCDD or 10 nM E2 alone or in combination or Me₂SO (DMSO) vehicle for 24 h, and EROD activity was measured as described under "Materials and Methods." Data are means ± S.D. (n = 4). Means with the same letter are not significantly different (p < 0.05).

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was incubated for 15 min at 25 °C. For competition with the specific unlabeled or mutant DRE probe or pure ER transformed with 1 nM E2 for 1 h at 25 °C (PanVera), the selected competitor (200-fold excess for DNA probes and 2 ng for ER) was incubated for 5 min prior to the addition of 32P-labeled DRE. The reaction was carried out in 20 mM HEPES, 5% glycerol, 100 mM KCl, 5 mM MgCl2, 0.5 mM dithiothreitol, 1 mM EDTA in a final volume of 25 μl. Reaction mixtures were loaded onto 5% polyacrylamide gel and run at 110 V in 0.09 M Tris, 0.09 M borate, 2 mM EDTA (pH 8.0). Gels were dried and protein-DNA binding was visualized by autoradiography.

Statistical Analysis—Results are presented as mean ± S.D. for at least four determinations for each treatment group. All experiments were carried out 2 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 ≤ 0.05 as the level of significance. Means with similar letters are not significantly different.

RESULTS

CYP1A1 Activity and mRNA Levels—Metabolism of ethoxyresorufin was used as a measure of CYP1A1-dependent activity in MCF-7 and Hepa 1c1c7 cells (Fig. 1). Following treatment of MCF-7 cells with 1 nM TCDD, there was a 4.9-fold increase in EROD activity compared with Me2SO-treated cells (Fig. 1A). Cotreatment of MCF-7 cells with 1 nM TCDD plus 1 nM E2 resulted in a 4.9-fold increase in EROD activity. E2 alone had no effect on EROD activity in MCF-7 cells. Likewise, treatment of Hepa 1c1c7 cells with 1 nM TCDD alone and in the presence of E2 resulted in a 5.6- and 6.1-fold increase, respectively, in EROD activity (Fig. 1B). In Hepa 1c1c7 cells E2 did not significantly induce EROD activity and E2 did not inhibit induction of EROD activity by TCDD in either cell line.

Competitive reverse transcriptase-PCR was used to determine the effect of E2 on induction of CYP1A1 mRNA levels by TCDD in MCF-7 and Hepa 1c1c7 cells. Consistent with the results obtained for induction of EROD activity, CYP1A1 mRNA levels were induced by 10 nM TCDD in both cell lines in the absence or presence of 10 nM E2 (Fig. 2, A and B). TCDD causes a 8.7- and 24.1-fold induction of mRNA levels in MCF-7 and Hepa 1c1c7 cells (lane 2), respectively. Me2SO and E2 alone did not induce CYP1A1 mRNA levels (lanes 1 and 3), and E2 did not significantly inhibit induction in either cell line (lanes 4).

Induction of CAT Activity in Transient Transfection Assays—To evaluate the effects of E2 on induction of CYP1A1 mRNA levels by TCDD in MCF-7 and Hepa 1c1c7 cells. Consistent with the results obtained for induction of EROD activity, CYP1A1 mRNA levels were induced by 10 nM TCDD in both cell lines in the absence or presence of 10 nM E2 (Fig. 3, A and B). TCDD caused a 6.0- and 9.5-fold increase in mRNA levels in MCF-7 and Hepa 1c1c7 cells (lane 2), respectively. Me2SO and E2 alone did not induce CYP1A1 mRNA levels (lanes 1 and 3), and E2 did not significantly inhibit induction in either cell line (lanes 4).
or presence of E2 (Fig. 3B) resulted in a 4-fold increase in CAT activity compared with MeSO-treated cells (compare lanes 2 and 4 with lane 1). E2 alone did not significantly affect CAT activity in Hepa 1c1c7 cells (lane 3).

Formation of a DRE-AhR Retarded Band—Nuclear extracts from MCF-7 and Hepa 1c1c7 cells treated with MeSO, TCDD, TCDD plus E2, or E2 were incubated with 32P-DRE and analyzed by gel electrophoretic mobility shift assays. Nuclear extracts from MCF-7 and Hepa 1c1c7 cells treated with TCDD formed a specifically bound retarded DRE-AhR complex (compare lanes 1 and 2 in MCF-7 (Fig. 4A) and Hepa 1c1c7 (Fig. 4B) cells. E2 alone had no effect on formation of this retarded band (lanes 3), and extracts from cells cotreated with TCDD plus E2 gave retarded bands with intensities (lanes 4) similar to that observed with extracts from cells treated with TCDD alone (lanes 2). AhR binding to the 32P-DRE could be competitively decreased by a 200-fold excess of unlabeled oligonucleotide (lanes 5 and 8) but not by a 200-fold excess of unlabeled mutated DRE (lanes 6 and 9). Incubation with excess ER had no effect on formation of the specifically bound retarded band (lanes 7 and 10).

**DISCUSSION**

Basal expression of CYP1A1 is low in hepatic and extrahepatic tissues of laboratory animals and in mammalian cells in culture. In many cells/tissues AhR agonists such as TCDD or 3-methylcholanthrene cause a marked induction of CYP1A1 gene expression, and the AhR-mediated mechanism of this response has been extensively investigated (17–19). Polymorphisms in the CYP1A1 gene and expression of this gene in various tissues are being used as prognostic factors for some cancers (20, 21), and this may be related, in part, to the important role of CYP1A1 in metabolic activation of structurally diverse carcinogens and toxins (20–22). Not surprisingly, there are numerous studies that have investigated cell-specific factors which regulate CYP1A1 expression. There is evidence that both cis-genomic sequences within the 5′-promoter region of the CYP1A1 gene and trans-acting factors may play an important role in modulating basal and inducible expression (23–27). Moreover, several other factors also inhibit induction of CYP1A1 and/or CYP1A2 gene expression, and they include interleukin 6 in human HepG2 cells (28), transforming growth factor-β in human A549 lung cancer cells (29), interleukin-1β, insulin, oxidative stress (e.g. H2O2), epidermal growth factor, transforming growth factor-α in mouse or rat hepatocytes (30–32), and the microtubule inhibitor nocodazole in mouse Hepa 1c1c7 cells (33). Some of these effects may be cell-specific since studies in this laboratory indicate that neither insulin nor epidermal growth factor inhibited induction of EROD activity by TCDD in MCF-7 cells.2 Kharat and Saatcioglu (13) recently reported that E2 inhibited induction of CYP1A1-dependent responses in Hepa 1c1c7 cells, and these results were in direct contrast to other reports in breast cancer cell lines where ER expression is required for Ah responsiveness (10, 12). The reported inhibitory effect of E2 also contrasted to results showing that 17α-ethynylestradiol enhanced induction of CYP1A1 in cultured chick embryo hepatocytes (34). Because of these conflicting reports this study has focused on determining the effects of E2 on induction of CYP1A1 and related activities in both mouse Hepa 1c1c7 and human MCF-7 cells. The results (Figs. 1–3) clearly demonstrate that E2 alone did not induce EROD activity and CYP1A1 mRNA levels of reporter gene activity, and in cells cotreated with E2 plus TCDD the presence of the hormone did not significantly affect transactivation. Moreover, E2 did not affect formation of the TCDD-induced AhR-DRE retarded band, which was detected by gel mobility shift assays (Fig. 4). These data demonstrate that E2 does not inhibit induction of CYP1A1 gene expression in Hepa 1c1c7 or MCF-7 cells, and the rationale for the differences between these results and a previous study (13) is unknown. Several studies indicate that the ER is important for AhR-mediated transactivation in breast cancer cells (10–12), and this was consistent with results showing that down-regulation of the ER in MCF-7 cells by the anti-estrogen ICI 164,384 was accompanied by loss of Ah responsiveness (35). It is possible that the ER sequesters corepressor protein(s) or acts as a coactivator for

2 D. Hoivik, K. Willett, C. Wilson, H. Liu, and S. Safe, unpublished results.
AhR-mediated gene expression in breast cancer cells, and current studies are utilizing in vitro transcription models to test this hypothesis.

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