

Analysis of the Antiestrogenic Activity of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in Human Ovarian Carcinoma BG-1 Cells

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ABSTRACT

We have used human ovarian carcinoma BG-1 cells to determine which steps in the pathway of estrogen signaling are disrupted by the aryl hydrocarbon receptor (AhR) ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). We report that inhibition of estrogen signaling occurs between 7 and 18 h after TCDD treatment and that this effect is not caused by a decrease in estradiol concentration. TCDD decreased estrogen receptor (ER) levels in cells grown in standard medium; however, in estrogen-stripped medium, ER (but not AhR) levels were dramatically reduced (~7-fold) but were not decreased further by TCDD. Because the absolute level of estradiol inducibility and inhibition by TCDD was similar in either medium,

decreases in ER are not responsible for the antiestrogenic effect. The AhR also did not bind to the estrogen-responsive element (ERE) *in vitro*, and ERE binding by nuclear ER complexes was not decreased by TCDD, indicating that the effect of TCDD does not involve direct competition between the AhR and ER for DNA binding. However, inhibition of protein synthesis by cycloheximide blocked the TCDD-induced inhibition of ER-dependent gene expression. Overall, our results are consistent with the action of a TCDD-induced protein at a step(s) after ER-DNA binding, most likely at the level of gene transcription.

In recent years, a great deal of attention has focused on compounds that can disrupt the estrogen homeostasis of an organism. The halogenated aromatic hydrocarbon (HAH) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) is reported to be a potent antiestrogen *in vivo* and in cells in culture. *In vivo* studies have shown that mice exposed to a nonlethal dose of TCDD exhibit a decrease in uterine weight, peroxidase activity, and epidermal growth factor binding, in addition to irregular estrus and overall reproductive failure (Umbreit et al., 1987). In addition, TCDD-dependent decreases in estrogen-dependent breast tumor growth in 7,12-dimethylbenz[*a*]anthracene-treated rats was observed (Holcomb and Safe, 1994). In cell cultures, TCDD has been shown to decrease estrogen-stimulated cell proliferation and secre-

tion of tissue plasminogen activator (Gierthy et al., 1987; Jana et al., 1999) and to inhibit the induction of several estrogen-dependent genes, such as cathepsin-D, pS2, progesterone receptor, and *c-fos* protooncogene (Duan et al., 1999).

TCDD and structurally related HAHs produce a variety of other toxic and biological effects, many of which are shown to be both tissue- and species-specific. Such adverse effects include an anorexic-like wasting syndrome, thymic atrophy, immunotoxicity, teratogenicity, tumor promotion, reproductive toxicity, dermal toxicity, and induction of gene expression including that of cytochrome P4501A1 (Poland, 1982; Poland and Knutson, 1982; Safe 1986; Denison et al., 1998). Biochemical and genetic studies have demonstrated that these effects are mediated by the aryl hydrocarbon receptor (AhR) (Denison et al., 1998; Whitlock, 1999). The AhR is a basic helix-loop-helix, ligand-dependent transcription factor that resides in the cytosol as part of a complex of proteins, including two 90-kDa heat-shock proteins and a 43-kDa protein termed hepatitis B virus X-associated protein (Carver and Bradfield, 1997; Ma and Whitlock, 1997; Meyer et al.,

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ABBREVIATIONS: HAH, halogenated aromatic hydrocarbon; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor; Arnt, AhR nuclear translocator; DRE, dioxin-responsive element; DMSO, dimethyl sulfoxide; ER, estrogen receptor; ERE, estrogen-responsive element; iDRE, inhibitory dioxin-responsive element; FBS, fetal bovine serum; ESM, estrogen-stripped medium; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GRIP1, glucocorticoid receptor-interacting protein-1; MEM, minimum essential medium; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline containing 1% Tween 20; RIP140, receptor-interacting protein 140; SRC-1A, steroid receptor coactivator-1A; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

1998). Upon ligand binding, the AhR translocates into the nucleus, dissociates from these proteins, and dimerizes with the AhR nuclear translocator (Arnt) protein. The binding of the heterodimer to its specific DNA recognition sequence, the dioxin-responsive element (DRE) (Denison et al., 1988a,b), results in protein recruitment and increased transcription of the adjacent gene (Whitlock, 1999).

The signaling pathway of the ER is similar in many ways to that of the AhR. The ER exists in at least two isoforms, termed ER α and ER β (Mosselman et al., 1996; Kuiper et al., 1997), and resides primarily in the nucleus, complexed with two 90-kDa heat-shock proteins, 70-kDa heat-shock protein, and an additional 55-kDa protein (Evans, 1988; Landel et al., 1994). After ligand binding, the ER dissociates from these proteins and forms a homodimer that then binds to an estrogen-responsive element (ERE), resulting in recruitment of coactivator proteins and a subsequent increase in gene transcription (Evans, 1988).

Several hypotheses regarding the mechanism(s) for the antiestrogenic action of TCDD have been proposed, including increased metabolism and clearance of estradiol by TCDD-induced enzymes (Spink et al., 1990) and down-regulation of ER mRNA and protein (DeVito et al., 1992; Wang et al., 1993; Tian et al., 1998; Wormke et al., 2000). It has also been reported that inhibition of the estrogen-induced cathepsin-D (Krishnan et al., 1995) and pS2 (Gillesby et al., 1997) genes by TCDD is caused by the presence of an inhibitory DRE (iDRE) located immediately adjacent to an estrogen-responsive region. Binding of the transformed TCDD-AhR complex to this iDRE is thought to interfere with DNA binding of the ER and/or ER-associated transcription factors, thus resulting in inhibition of ER-dependent gene transcription (Krishnan et al., 1995; Gillesby et al., 1997; Safe, 2001). Although the iDRE seems to play a role in the antiestrogenic action of TCDD on cathepsin-D and pS2 gene expression in MCF-7 cells, the ability of TCDD to inhibit estrogen-induced gene expression from EREs lacking an iDRE (Nodland et al., 1997; Legler et al., 1999) suggests the existence of multiple, perhaps gene- and tissue-specific, mechanisms for TCDD-dependent antiestrogenicity.

Recently, the role of coactivator proteins in mediating the transactivation functions of the ER and AhR has been examined (Shibata et al., 1997; Kumar and Perdew, 1999; Kumar et al., 1999; Klinge, 2000). Coactivator proteins such as RIP140 and SRC-1A seem to enhance both ER- and AhR-dependent transcription by bridging the DNA-bound receptor and the basal transcriptional machinery, and also perhaps by altering chromatin structure as a result of their ability to affect histone acetylation (Shibata et al., 1997; Spencer et al., 1997; Klinge, 2000). Given the similarities in molecular mechanisms of both receptors, it has been suggested that they compete for a limited pool of coactivators and that sequestration by the AhR complex of one or more proteins, required for ER function, may be responsible for the antiestrogenic effect of TCDD.

We have recently developed a novel recombinant human ovarian (BG-1) cell line that contains a stably transfected ERE-driven luciferase reporter gene that responds to estrogens in a time- and dose-dependent manner (Rogers and Denison, 2000b). Using this novel cell line, we demonstrate the ability of TCDD to inhibit the estrogen-induced expression of the luciferase reporter gene as well as the endogenous

pS2 gene. Detailed mechanistic analysis of each step in the pathway of estrogen signaling suggests that the predominant inhibitory action of TCDD in BG-1 cells occurs at a step after ER-DNA binding and most probably results from the action of a TCDD-induced protein.

Experimental Procedures

Materials. Molecular biological enzymes were purchased from New England Biolabs (Beverly, MA) or Invitrogen (Carlsbad, CA). Polybrene was obtained from Aldrich Chemical Co. (Milwaukee, WI), G418 was obtained from Invitrogen, and diethylstilbestrol, 17 β -estradiol, and cycloheximide were obtained from Sigma Aldrich (St. Louis, MO). TCDD and 4'-amino-3'-methoxyflavone were gifts from Dr. S. Safe (Texas A&M University, College Station, TX).

Plasmids. The estrogen-responsive luciferase reporter plasmid, pGudLuc7ere, was constructed as described previously. This plasmid contains the luciferase gene and mouse mammary tumor virus promoter under control of four EREs (Rogers and Denison, 2000b). The pcDNA3.1- β mAhR plasmid was a kind gift from Dr. Oliver Hankinson (UC Los Angeles) and contains a constitutively expressed full-length mouse AhR cDNA under the translational control of the β -globin 5'-untranslated region in the plasmid pcDNA3.1 (Invitrogen, Carlsbad, CA). The pcDNA3.1-mArnt plasmid was obtained from Dr. Carol Jones (University of California, Davis, CA), and it contains the murine Arnt cDNA (from Dr. James P. Whitlock, Jr. (Stanford University) inserted immediately downstream of the CMV promoter in the plasmid pcDNA3.1.

Cell Culture and Transient Transfection Experiments. BG-1 (human ovarian carcinoma) cells were kindly provided by Dr. George Clark (Xenobiotic Detection Systems Inc., Durham, NC). BG1Luc4E₂ cells were previously generated by stably cotransfecting BG-1 cells with pSV2Neo and pGudLuc7ere (Rogers and Denison, 2000b). Cells were grown in standard medium [α -minimum essential medium (α MEM)] (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA) at 37°C in an atmosphere of 5% CO₂ and 85% humidity. BG1Luc4E₂ cells were maintained in standard medium containing 0.4 mg/ml geneticin (G418). For transient transfections, cells were grown in six-well plates in either standard medium (α MEM with 10% FBS) or for 6 days in estrogen-stripped medium (ESM, phenol red-free MEM) (Sigma-Aldrich) containing 5% dextran-coated charcoal-treated FBS (Hyclone, Logan, UT), changing the medium daily. At 60% confluence the cells were transiently transfected with the indicated amount of the desired plasmid, using Polybrene, as described previously (Garrison et al., 1996), and after 6 h, cells were shocked for 90 s in medium containing 15% glycerol. Cells were then allowed to grow for 48 h followed by chemical treatment for the indicated time, after which cells were lysed and luciferase activity was determined as described below.

Chemical Treatment and Measurement of Luciferase Activity in Stable Transfectants. Cells were plated in 12-well or 24-well plates and cultured in either ESM for 6 days with daily medium changes or in standard medium. Unless otherwise stated, the cells were exposed to the indicated chemicals for 24 h (final solvent concentrations of 0.1%, v/v). The medium was then removed, the plates were rinsed with phosphate-buffered saline (PBS), and cells were lysed with 150 μ l of lysis buffer (Promega, Madison, WI). Lysed samples were collected, and the cell debris was pelleted by centrifugation. Luciferase activity in 45 μ l of cleared lysate was measured either in a Dynatech ML3000 or an Anthos Lucy2 microplate luminometer (delay time of 5 s and an integration time of 10 s) after the addition of 50 μ l of luciferase reagent (Promega). Luciferase activity was normalized to the protein concentration of the cell lysate using fluorescamine (Kennedy et al., 1995) and bovine serum albumin as the protein standard. Briefly, 100 μ l of fluorescamine (500 μ g/ml in acetonitrile) (Molecular Probes, Eugene, OR) was added to each

microplate well, and the plate was covered with foil and agitated for 20 min. Fluorescence was measured in a Fluostar plate reader with excitation and emission wavelengths of 390 nm and 460 nm, respectively.

Preparation of Cellular Proteins. For whole cell extracts, cells were grown to 90% confluence in ESM for 6 days, changing the medium daily, or in standard medium, and then treated for 24 h. Cells were then washed twice with ice-cold PBS, collected in 2 ml of PBS per 10-cm plate, pelleted, and resuspended in 0.5 ml of homogenization buffer per plate [10 mM Tris, pH 7.4, 1 mM EDTA, 2 mM DTT, 10% (v/v) glycerol, 0.5 M NaCl, and one protease inhibitor tablet (Roche Applied Science, Mannheim, Germany) per 10 ml of buffer]. The homogenate was centrifuged at 100,000g for 1 h at 4°C, and the resulting whole cell extract was stored at -80°C.

For nuclear and cytosolic protein, cells treated for 24 h with the indicated chemical or solvent were trypsinized, pelleted, and homogenized with a Dounce homogenizer in 1 ml of MDH (3 mM MgCl₂, 1 mM DTT, 25 mM HEPES, pH 7.5, and one Roche protease inhibitor tablet per 10 ml of buffer) per 10-cm plate of cells. The homogenates were then centrifuged at 1,000g for 5 min at 4°C, and the supernatant was centrifuged further at 100,000g for 1 h at 4°C to obtain the cytosolic fraction. The pellet from the 1,000g spin was washed twice by resuspending it in 1 ml of MDH (MDH + 0.1 M KCl) per 10-cm plate and recentrifuged at 1,000g for 2 min at 4°C. The washed pellet was resuspended in 25 µl of HDKG [25 mM HEPES, pH 7.5, 1 mM DTT, 0.4 M KCl, 10% (v/v) glycerol, and one protease inhibitor tablet per 10 ml of buffer) per plate and incubated for 20 min on ice with vortexing every 5 min. The incubation was centrifuged at 12,000g for 20 min at 4°C, and the resulting supernatant was centrifuged at 100,000g for 1 h at 4°C to obtain the final nuclear protein extract.

Western Immunoblotting. For Western blotting, whole cell extracts, cytosol or nuclear protein, were separated by SDS-polyacrylamide gel electrophoresis, followed by electroblotting to nitrocellulose membranes. For immunostaining, the blots were blocked in PBS containing 1% Tween 20 (PBST) and 5% nonfat dry milk for 1 h at room temperature. The blots were then washed in PBST and incubated with ERα antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a concentration of 0.1 µg/ml in PBST with 1% bovine serum albumin, for 1 h at room temperature. Blots were rewashed and incubated with a 1/12,500 dilution of horseradish peroxidase-conjugated goat anti-rabbit antibody (Sigma-Aldrich) in PBST with 1% bovine serum albumin, for 1 h at room temperature. The blots were washed again and exposed to Chemiluminescent Reagent Plus (PerkinElmer Life Sciences, Boston, MA) and exposed to X-ray film for 20 s.

Gel Retardation Assay. Complementary DNA oligonucleotides containing a single ERE from the chicken vitellogenin A2 gene (ERE) (5'-GATCTGACTCCGGTACGCTGACCAGGAATATTG-3') or DRE3 from the 5' flanking region of the CYP1A1 gene (DRE) (5'-GATCCGGAGTTGCGTGAGAAGAGCCA-3') were synthesized by the Macromolecular Structure Facility, Michigan State University. Oligonucleotides were purified, annealed, and end-labeled with [γ -³²P]ATP as described previously (Denison et al., 1988a). Seven micrograms of nuclear extract were incubated in HEDG buffer [25 mM HEPES, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol] with 1.8 µg of poly(dI-dC) for 15 min at room temperature, followed by incubation for another 15 min with 150,000 cpm ³²P-labeled oligonucleotide. Samples were separated on a 4% nondenaturing polyacrylamide gel, with buffer recirculation, and the resulting protein-DNA complexes were visualized by autoradiography and quantified using a PhosphorImager SI (Molecular Dynamics, Sunnyvale, CA).

Gel Retardation Assay using Transcription/Translation Lysates. Mouse AhR and Arnt proteins were synthesized *in vitro* using the Reticulocyte Lysate TnT (transcription/translation) system (Promega) and the pcDNA3.1-8mAHR and pcDNA3.1-mArnt vector templates according to the manufacturer's protocol. The AhR was transformed by incubating 1.5 µl each of AhR and Arnt reticulocyte

lysates with HEDG and 20 nM TCDD or DMSO (10%, v/v), in a final reaction volume of 10 µl, for 2 h at room temperature. The TCDD-treated AhR reaction mixture was incubated with 15 µl of oligo buffer [42 mM HEPES, 0.33 M KCl, 17% glycerol (v/v), 8.3 mM DTT, 16.7 mM EDTA, 0.125 mg/ml CHAPS, 337.5 ng poly(dI-dC)] for 15 min at room temperature, followed by addition of 150,000 cpm of ³²P-labeled oligonucleotide and further incubation at room temperature for 15 min. Protein-DNA complexes were separated on a 4% nondenaturing polyacrylamide gel and visualized as described above.

ER Ligand Binding Analysis [³H] Estradiol binding was determined using dextran-coated charcoal. Whole cell extracts (0.5 mg, 1 mg/ml) from BG1Luc4E₂ cells were incubated with 2 nM [³H]estradiol in the absence or presence of a 100-fold molar excess of diethylstilbestrol for 2 h at 4°C. Extracts were then mixed with 0.5 ml of a charcoal pellet [0.5 mg charcoal (Norit A)/0.05 mg dextran (average molecular weight 127,000)/ml water] and incubated for 15 min at 4°C, followed by centrifugation at 3,500 rpm for 15 min. Radioactivity in an aliquot of the supernatant (300 µl) was then determined by liquid scintillation counting. Specific binding of [³H]estradiol to the ER was computed by subtracting the amount of [³H]estradiol bound in the presence of diethylstilbestrol from the amount of [³H]estradiol bound in the absence of competitor. Specific binding values were expressed as femtomoles of [³H]estradiol bound per milligram of protein.

Northern Blot Analysis. Total RNA from treated cells was isolated using the RNeasy Mini Kit from QIAGEN (Valencia, CA). Total RNA was separated on a 1% agarose/formaldehyde gel, transferred overnight to a nylon membrane, baked at 80°C for 1 h, and UV-crosslinked. Blots were prehybridized for 1 h at 65°C in 10 ml of hybridization solution (7% SDS, 0.25 M sodium phosphate, 1 mM EDTA) and then probed overnight at 65°C with randomly primed cDNA fragments (Amersham Biosciences, Piscataway, NJ) labeled with [α -³²P]dCTP at 1 × 10⁶ cpm/ml of hybridization solution. Blots were then washed three times for 5 min with 2× standard saline citrate (0.3 M NaCl, 30 mM sodium citrate, pH 7.0), 0.1% SDS at 65°C, and once for 15 min with 0.5× standard saline citrate, 0.1% SDS at 65°C. The resulting blots were visualized by autoradiography and quantified using a Molecular Dynamics PhosphorImager SI. Probes used in Northern blots were obtained as follows: GAPDH was obtained from Dr. Larry Hjelmeland (University of California, Davis, CA), pS2 from Dr. William Helferich (University of Illinois at Urbana Champaign), human AhR from Dr. Patricia Harper (Hospital for Sick Children, Toronto, ON, Canada), human ER was from Dr. Tim Zacharewski (Michigan State University, East Lansing, MI), and human CYP1A1 was from the American Type Culture Collection (Manassas, VA).

Results

TCDD Inhibits Estrogen Signaling in BG-1 and BG1Luc4E₂ Cells. Growth of BG1Luc4E₂ cells in standard medium, which contains phenol red and 10% FBS, results in high constitutive luciferase activity that is not increased further by estradiol addition and that has previously been attributed to estrogens (including phenol red and FBS) in the medium (Rogers and Denison, 2000b). This constitutive luciferase activity is decreased by TCDD, with maximum inhibition of 30 to 60% with 10 nM TCDD (Fig. 1). To show a more direct effect of TCDD on estrogen signaling, the cells were grown in ESM (phenol red-free MEM containing charcoal-stripped FBS) for several days to substantially reduce background luciferase activity (Rogers and Denison, 2000b). Treatment of these cells with 1 nM estradiol results in 50- to 100-fold induction of luciferase over background, whereas cotreatment with 1 nM estradiol and 10 nM TCDD results in

a 30 to 60% decrease in estrogen-induced luciferase activity (Fig. 1). Figure 1 also demonstrates not only that the absolute level of maximal luciferase activity is similar between BG1Luc4E₂ cells grown in standard medium and ESM but also that TCDD reduces luciferase activity to the same degree in cells grown in either medium. A similar degree of inhibition by TCDD is also observed in transient transfection studies where the parent BG-1 cells, grown in ESM for several days, were transfected with pGudLuc7ere, the same plasmid that has been stably integrated into the recombinant BG1Luc4E₂ cells (data not shown). These results confirm that the TCDD-dependent inhibition of estradiol-dependent gene expression in the stably transfected cells is caused not simply by its site of integration in genomic DNA or to bases adjacent to the integrated plasmid but by an effect on the vector itself.

The AhR dependence of the effect was determined by using the AhR antagonist 4'-amino-3'-methoxyflavone (Lu et al., 1995), which blocked the TCDD-dependent inhibition of estrogen-induced luciferase (data not shown). To determine how rapidly TCDD inhibits estrogen-induced luciferase activity, we analyzed the time course of TCDD inhibition in BG1Luc4E₂ cells grown in ESM. Although there were some inconsistencies between experiments with respect to the exact time that inhibition was first observed, the onset of TCDD-dependent inhibition generally occurred between 7 and 18 h after treatment (Fig. 2). The time course of inhibition was also performed using 10 nM TCDD, and similar results were obtained (data not shown). In addition, dose-response data for the antiestrogenic effect of TCDD indicates that 10 nM TCDD seems to be only slightly better at inhibiting estrogen signaling in these cells than 1 nM TCDD (data not shown). These results suggest that the effect of TCDD on estrogen-induced luciferase activity was not a direct, primary event, which would have been observed much earlier, but could probably result from an indirect, secondary response.

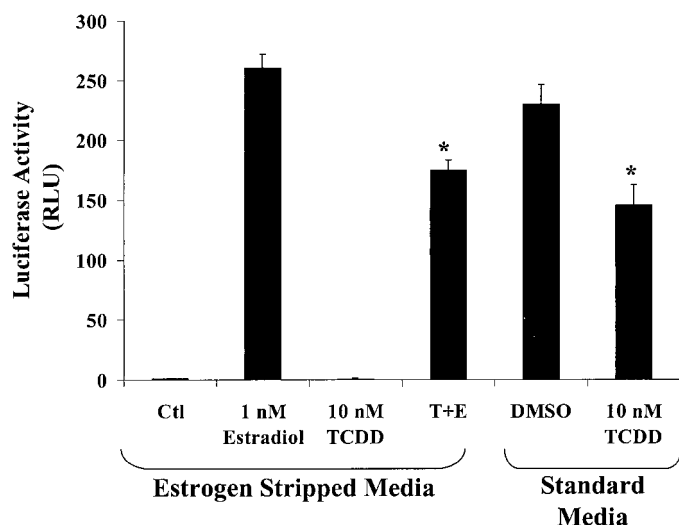


Fig. 1. Inhibition of luciferase activity by TCDD in BG1Luc4E₂ cells. Plates of BG1Luc4E₂ cells were grown in standard medium or in ESM and were treated for 24 h with the indicated chemicals. Luciferase activity was determined as described under *Experimental Procedures*. Values represent mean \pm S.D. of six determinations, and the asterisks indicate that the value is significantly ($p < 0.05$) different from the respective 1 nM estradiol or DMSO control as determined by Student's *t* test. This experiment was performed at least twice with similar results. RLU, relative light units.

Inhibition of Estrogen Signaling by TCDD Is Not Mediated by Reduction in the Level of Estradiol. Previous studies have suggested that metabolic reduction in the concentration of estradiol by TCDD-induced enzymes such as CYPs1A1 and 1B1 is responsible for the antiestrogenic effect of TCDD (Spink et al., 1990). To test this possibility in our cells, we determined the effect of adding excess estradiol on the observed inhibition by TCDD. Increasing the concentration of estradiol in the medium by 1000-fold (to 1 μ M) did not block the inhibitory effect of TCDD (Fig. 3). These results suggest that the antiestrogenic effect of TCDD in BG-1 cells is not simply caused by a reduction in estradiol concentration resulting from enhanced estrogen metabolism by TCDD-induced enzymes.

TCDD Decreases ER Protein and mRNA in BG1Luc4E₂ Cells. The ability of TCDD to reduce intracellular ER levels has been reported; however, this effect does not occur in all systems. Tian et al. (1998) have reported significant TCDD-induced decreases in ER mRNA, as determined by reverse transcription-polymerase chain reaction, in liver, ovary, and uterus from CD-1 mice, whereas Gierthy et al. (1996) reported that TCDD treatment did not affect ER mRNA levels in MCF-7 cells. To examine the effect of TCDD on the ER in BG-1 cells, we carried out ligand binding, Western, and Northern analyses. In our previous studies, we have reported that BG1Luc4E₂ cells contain substantial levels of ER α but do not contain detectable levels of ER β as determined by Western blot (Rogers and Denison, 2000b). Radioactive ligand binding analysis of whole cell extracts from DMSO- and TCDD-treated cells, grown in standard medium, shows a 40% reduction in [³H]estradiol-specific binding with TCDD treatment (Fig. 4A). A similar degree of inhibition of [³H]estradiol binding to cytosolic proteins from TCDD-treated cells was also observed (data not shown). These results address ligand-binding activity but do not indicate whether the decrease in binding is caused by a reduction in ER

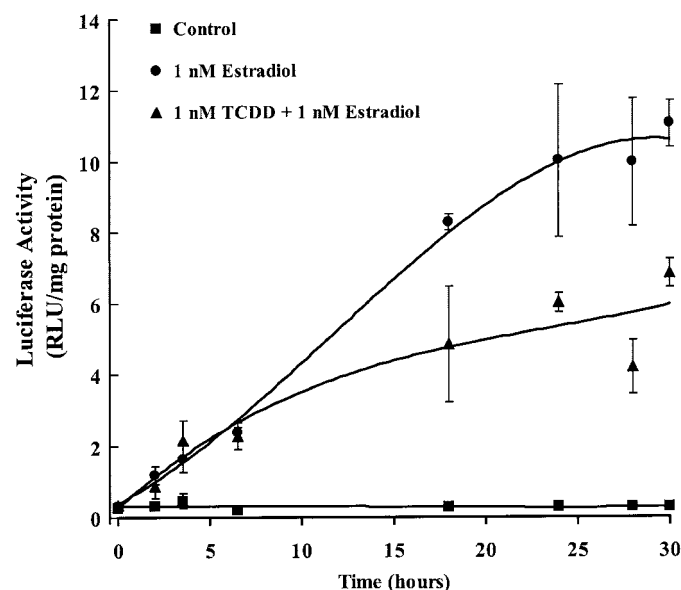


Fig. 2. Time course of inhibition of estrogen-induced luciferase activity by TCDD in BG1Luc4E₂ cells grown in ESM. Plates of BG1Luc4E₂ cells were grown for 6 days in ESM, with daily medium changes, and then treated with the indicated chemicals for increasing amounts of time. Luciferase activity was determined as described under *Experimental Procedures*. Values represent mean \pm S.D. of four determinations. This experiment was performed at least four times with similar results.

levels or by an effect on ER ligand-binding activity itself. To confirm an effect of TCDD on ER protein levels, Western blot analysis was carried out. These experiments demonstrate that treatment of cells grown in standard medium for 24 h with 10 nM TCDD resulted in a similar decrease in the amount of cytosolic and nuclear ER α protein compared with DMSO-treated cells (Fig. 4B). To determine whether the TCDD-induced decrease in ER α protein was occurring at the level of the ER α mRNA, we carried out Northern blot analysis. These experiments revealed that treatment of BG1Luc4E₂ cells with TCDD for 24 h also resulted in a significant (18–25%) reduction of ER α mRNA (Fig. 4C). Although this degree of inhibition is less than that observed at the ligand binding and protein levels, it was consistent between experiments.

Growth of BG1Luc4E₂ Cells in ESM Results in a Significant Decrease in ER α . Given that the relative reduction in ER ligand-binding activity is similar to the inhibition observed with luciferase induction, it is possible that the reduction in cellular ER could be responsible for the TCDD-dependent decrease in estrogen signaling in BG1Luc4E₂ cells. However, both ER ligand binding (Fig. 5A) and Western blot analyses (Fig. 5B) demonstrate a substantial decrease (~7-fold) in the amount of ligand-binding activity and ER α protein in cells grown in ESM for several days compared with those grown in standard medium. Northern blot analysis confirms that this decrease in ER α protein probably results from the significant reduction in mRNA levels (60 to 70%), a change that occurs at the level of gene transcription and/or message stability (Fig. 5C). Transfer of cells grown in ESM for 4 days back into standard medium results in full restoration of ER α levels in 3 to 4 days (data not shown). Thus, the constitutive expression of ER α in BG-1 cells seems to be dependent upon a factor(s) absent in the charcoal-stripped serum or medium (ESM). In addition, although we detect only low levels of ER α in cells grown in ESM, these levels do not seem to be decreased by TCDD treatment, as seen by Western and ligand binding analyses (Fig. 5, A and B). Be-

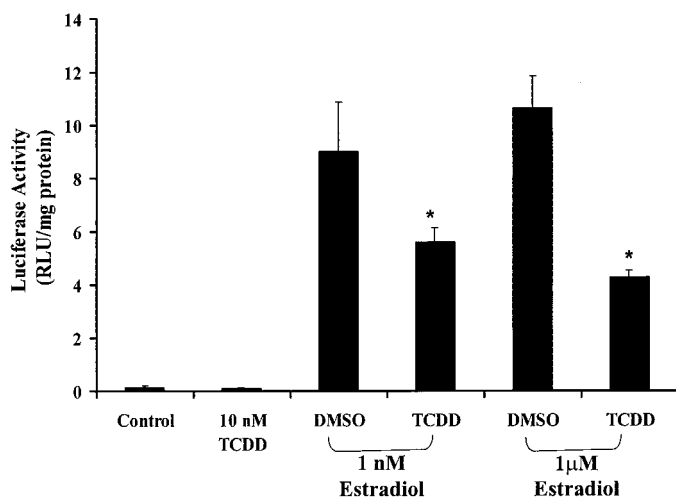


Fig. 3. Addition of excess estradiol does not alter the TCDD-induced inhibition of luciferase activity in BG1Luc4E₂ cells. Cells were grown for 5 days in ESM, with daily medium changes, and then treated for 24 h with the indicated compounds. Luciferase activity was determined as described under *Experimental Procedures*. Values represent mean \pm S.D. of at least triplicate determinations, and the asterisks indicate that the value is significantly different ($p < 0.05$) from 1 nM estradiol as determined by Student's t test. This experiment was performed at least four times with similar results.

cause TCDD represses estrogen-inducible gene expression in cells grown in either medium, but only decreases ER α levels in cells grown in standard medium (i.e., those with high ER

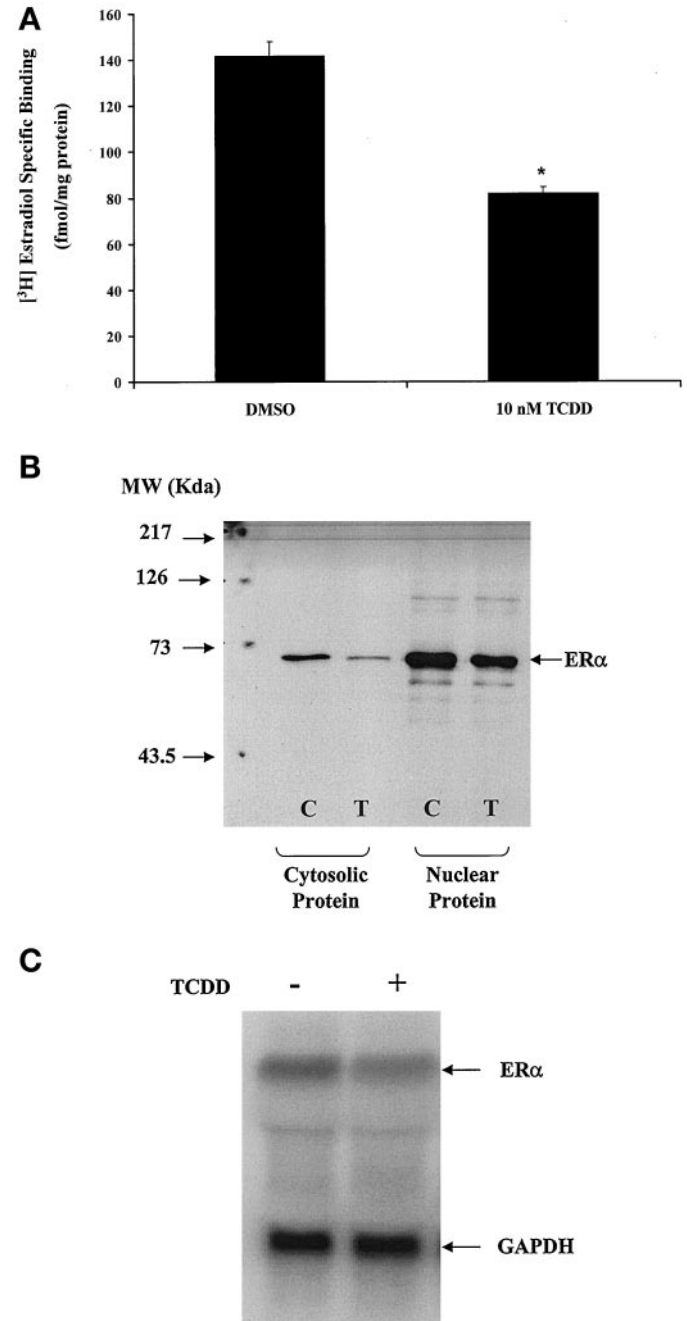


Fig. 4. TCDD decreases ER ligand binding, protein, and mRNA in BG1Luc4E₂ cells. Cells were grown in standard medium and treated for 24 h with DMSO (C) or 10 nM TCDD (T). Protein and RNA were isolated as described under *Experimental Procedures*. A, [³H]estradiol-specific binding in whole cell extracts of BG1Luc4E₂ cells. The asterisk indicates that the value is significantly different ($p < 0.05$) from DMSO as determined by Student's t test. This experiment was performed at least four times with similar results. B, Cytosolic or nuclear protein extracts (5 μ g) were analyzed by immunoblotting for ER α protein. This experiment was performed in duplicate with similar results, and a representative blot is shown. C, total RNA (15 μ g) was analyzed by Northern blot, and the membrane was probed with the indicated radiolabeled cDNA fragments. The bands were quantified using a Molecular Dynamics PhosphorImager, and ER α values were normalized to GAPDH. This experiment was performed in triplicate with similar results (C = 0.44 ± 0.02 , T = 0.36 ± 0.01), and a representative blot is shown.

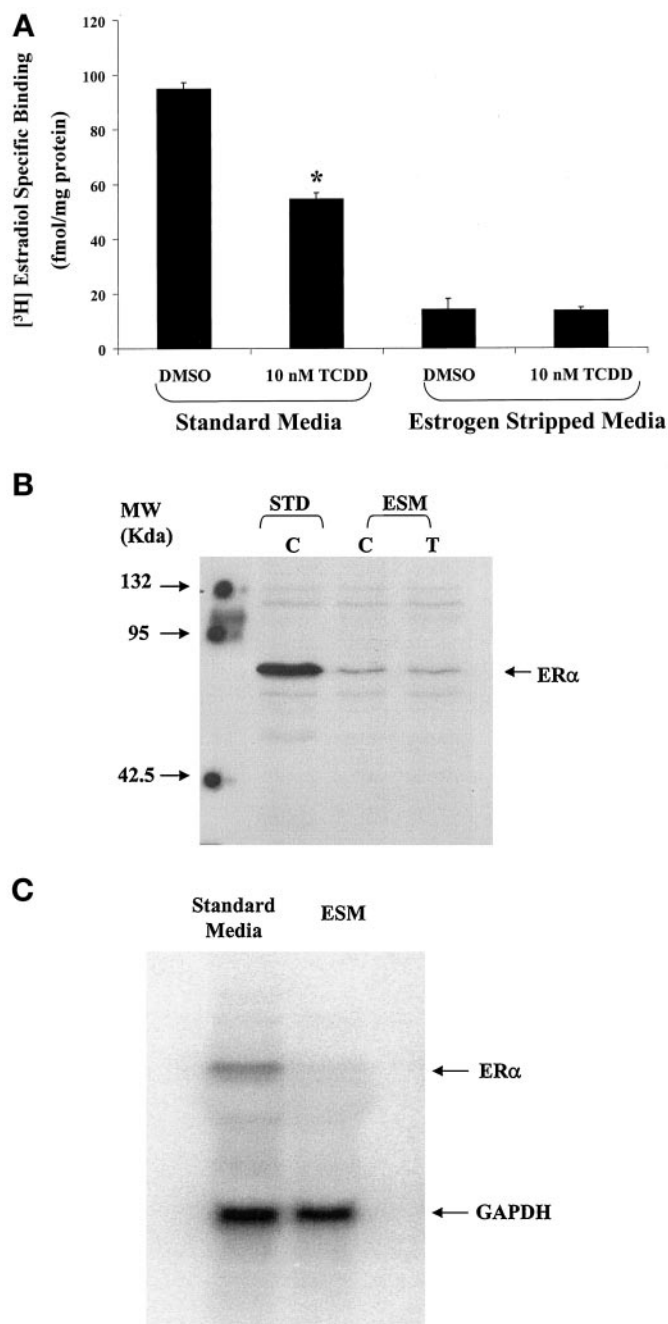


Fig. 5. Growth of BG1Luc4E₂ cells in ESM down-regulates the ER, which is not further decreased by TCDD. A, comparison of [³H]estradiol-specific binding in whole cell extracts of BG1Luc4E₂ cells grown in either standard medium or ESM and treated for 24 h with DMSO or 10 nM TCDD. Whole cell extract isolation and [³H]estradiol-specific binding were determined as described under *Experimental Procedures*. Values represent mean \pm S.D. of triplicate determinations, and the asterisk indicates that the value is significantly different ($p < 0.05$) from the respective DMSO control as determined by Student's *t* test. B, comparison of ER α protein content in BG1Luc4E₂ cells grown in either standard medium or ESM. Cells were treated for 24 h with DMSO (C) or 10 nM TCDD (T). Whole cell extracts (5 μ g) were isolated and analyzed by immunoblotting for ER α protein as described under *Experimental Procedures*. This experiment was performed at least twice with similar results. C, comparison of ER α mRNA in BG1Luc4E₂ cells grown in either standard medium or ESM. RNA was isolated as described under *Experimental Procedures*. Total RNA (15 μ g) was analyzed by Northern blot, and the membrane was probed with the indicated radiolabeled cDNA fragments. The bands were quantified using a Molecular Dynamics PhosphorImager, and ER α values were normalized to GAPDH. This experiment was performed in duplicate with similar results, and a representative blot is shown.

levels), the antiestrogenic effect must not be mediated by an effect on ER α levels.

Growth of BG1Luc4E₂ Cells in ESM Does Not Decrease AhR Expression or Function. The above results demonstrate that TCDD decreases estrogen responsiveness of cells grown in ESM without altering ER α levels in these cells. To determine whether growth of cells in ESM also had a negative effect on the AhR or AhR-dependent gene expression, we determined the levels of AhR- and TCDD-induced CYP1A1 mRNA in cells grown in ESM and standard medium. Northern blot analysis revealed no decrease in AhR mRNA levels (Fig. 6A) or TCDD inducibility of CYP1A1 (Fig. 6B) in cells grown in ESM compared with standard medium. Thus the overall ratio of AhR to ER in cells grown in ESM is substantially greater (~ 7 -fold) than those in standard medium. The fact that the degree of TCDD antiestrogenicity does not increase in cells grown in ESM, even though the AhR/ER ratio dramatically increases, argues against the antiestrogenic activity of TCDD in BG-1 cells being simply

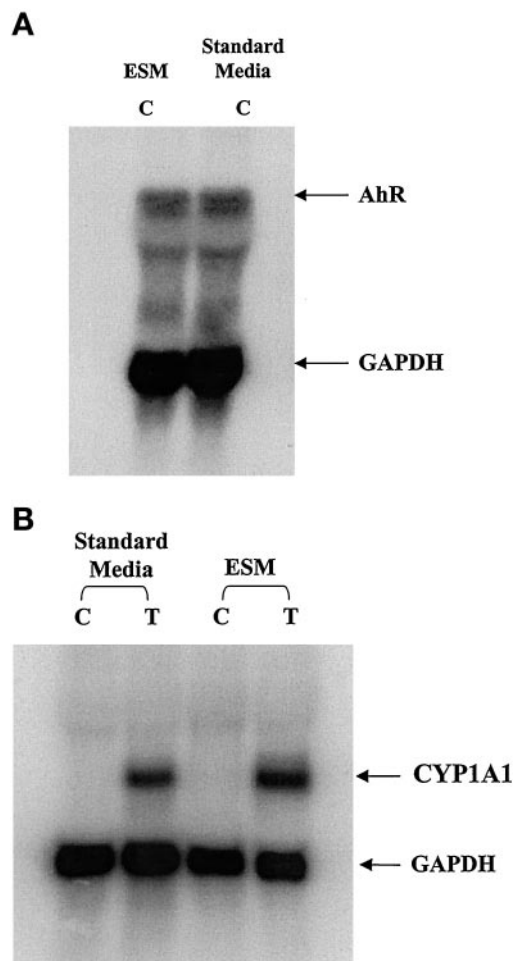


Fig. 6. Growth of BG1Luc4E₂ cells in ESM does not decrease AhR mRNA levels or the TCDD-dependent induction of CYP1A1 mRNA. Cells were grown in standard medium or in ESM for 6 days and treated for 24 h with DMSO (C) or 10 nM TCDD (T). RNA was isolated as described under *Experimental Procedures*. Total RNA (15 μ g) was analyzed by Northern blot, and the membranes were probed with radiolabeled AhR (A) or CYP1A1 (B) cDNA fragments. The bands were quantified using a Molecular Dynamics PhosphorImager, and AhR and CYP1A1 values were normalized to GAPDH. Samples were run in duplicate with similar results, and a representative blot is shown.

caused by direct competition between the AhR and the ER for nuclear factors and/or DNA binding.

TCDD Does Not Inhibit Binding of ER to DNA. A critical step in the pathway of estrogen signaling is the binding of the ER complex to the ERE. To examine whether TCDD could directly disrupt ER-DNA binding, we used gel retardation analysis. Our initial step was to determine whether the AhR could directly bind the ERE, which was responsible for estrogen signaling in our reporter system. Although we observed that reticulocyte lysate-expressed TCDD-AhR-Arnt complex could bind to a [32 P]DRE oligonucleotide, no TCDD-inducible protein-DNA complex was formed using a 32 P-labeled oligonucleotide containing the ERE from the chicken vitellogenin A2 gene (Fig. 7A). We also examined the ability of nuclear proteins from control and TCDD-treated BG1Luc4E₂ cells, grown in standard medium, to bind these oligonucleotides. Although a TCDD-induced protein-DNA band was formed with the [32 P]DRE oligonucleotide, albeit a small amount, no TCDD-inducible AhR-DNA complex was formed with the chicken vitellogenin ERE (Fig. 7B). These results clearly indicate that the AhR does not bind, at least in vitro, to the ERE we have used in our system. Formation of the ER-ERE complex was unaffected by TCDD treatment (Fig. 7B), indicating that TCDD does not reduce ER binding to DNA, even though total cellular ER levels were decreased by approximately 40% (Fig. 4, A and B). Competitive gel retardation analysis using wild-type and mutant EREs demonstrated that the single protein-ERE complex indicated as the ER complex in Fig. 7B has the DNA binding specificity of the ER (data not shown).

Over-Expression of Selected Coactivator Proteins Does Not Eliminate the Antiestrogenic Effect of TCDD in BG-1 Cells. Previous studies have demonstrated the role of several coactivators in ER-dependent gene expression (Shibata et al., 1997; Klinge, 2000). Although our results suggest that the antiestrogenic effect of TCDD is not simply caused by the ability of the AhR to compete for, and sequester, ER-binding factors, it is possible that TCDD could alter the function of coactivators by some other mechanism. Therefore, to examine the role of coactivator proteins on the inhibition of estrogen signaling by TCDD, we over-expressed several of these factors, including receptor-associated coactivator-3, GRIP1, SRC-1A, RIP140, and p300, all of which have been shown by others to interact with either the ER, the AhR, or both (Shibata et al., 1997; Kumar and Perdew, 1999; Kumar et al., 1999; Klinge, 2000). BG-1 cells were grown in ESM for three days and cotransfected with pGudLuc7ere and the indicated coactivator, and then treated for 24 h. Although all of the coactivators we tested increased background luciferase activity by 2- to 8- fold, none of these factors could overcome the antiestrogenic effect of TCDD (Table 1).

Inhibition of Protein Synthesis Blocks the TCDD-Dependent Inhibition of Estrogen-Induced Gene Expression. To determine whether the antiestrogenic action of TCDD in BG1Luc4E₂ cells is mediated directly by the AhR as a primary event or indirectly by a TCDD-induced factor, BG1Luc4E₂ cells were grown in standard medium, and then protein synthesis was inhibited with cycloheximide. Cells were treated for 24 h to ensure the maximal effect of TCDD; however, no visible signs of toxicity were observed after exposure to cycloheximide for this amount of time. Although TCDD decreased background luciferase mRNA by 35 to 40%,

when cells were treated for 24 h with cycloheximide luciferase mRNA levels from TCDD-treated cells were 92% of the respective cycloheximide control (Fig. 8A). However, treatment of cells with cycloheximide increased background luciferase mRNA. Thus, although TCDD-dependent inhibition of luciferase gene expression was blocked by cycloheximide, it is possible that the "superinduction" phenomenon could have negated or masked the TCDD effect. We therefore examined the effect of cycloheximide on endogenous pS2 gene expression, to determine whether the same effect was observed. Cycloheximide did not superinduce pS2 mRNA levels in cells

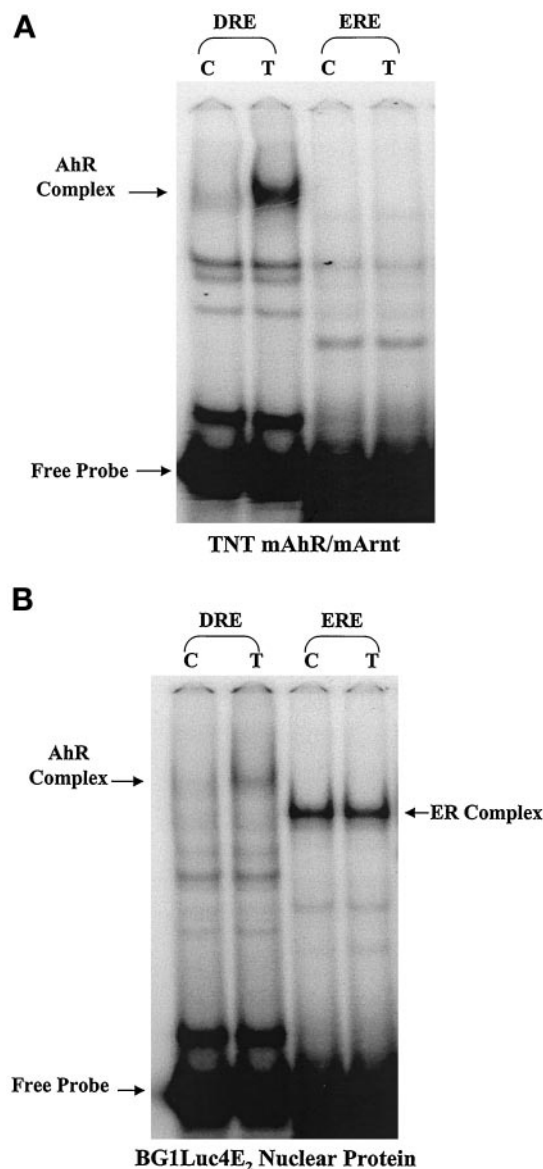


Fig. 7. The AhR does not bind the ERE in vitro, and TCDD does not decrease the nuclear ER complex binding to the ERE in vitro. A, mouse AhR and Arnt proteins were synthesized in vitro, incubated with DMSO (C) or 20 nM TCDD (T), and analyzed in gel retardation analysis with 32 P-labeled DRE or ERE oligonucleotides, as described under *Experimental Procedures*. This experiment was performed in duplicate with similar results, and a representative blot is shown. B, BG1Luc4E₂ cells were grown in standard medium and treated for 24 h with DMSO (C) or 10 nM TCDD (T). Nuclear protein was isolated, and gel retardation analysis was carried out with 32 P-labeled DRE or ERE, as described under *Experimental Procedures*. This experiment was performed in duplicate with similar results, and a representative blot is shown.

grown in standard medium but resulted in a small decrease in control (DMSO) mRNA levels (Fig. 8B). In the absence of cycloheximide, TCDD reduced pS2 mRNA levels to 60 to 70% of that of DMSO-treated cells, whereas in the presence of cycloheximide, TCDD had no significant effect on pS2 mRNA levels (95–100% of control). These data are consistent with a role for a TCDD-induced protein as being responsible for mediating the inhibition of estrogen-dependent pS2 and luciferase gene expression in BG1Luc4E₂ cells.

Discussion

It has been well established that TCDD and structurally related HAHs are antiestrogenic both *in vivo* and in a variety of cell lines; however, the precise mechanism(s) for this inhibition remains unclear. One way in which TCDD could reduce estrogenic activity is to reduce the amount of available estrogen within a system. Spink et al. (1990) demonstrated that cotreatment of MCF-7 breast cancer cells with TCDD and estradiol resulted in rapid reductions in both intracellular and extracellular estradiol compared with estradiol treatment alone. Microsomes from these cells also showed an increase in aryl hydrocarbon hydroxylase activity (a relatively specific indicator of CYP1A1 activity) and hydroxylation of estradiol. In the present study, we addressed this issue by examining the effect of adding excess estradiol to the system. If inhibition of estrogen signaling were simply caused by a decrease in available estradiol, then the addition of 1000-fold excess estradiol would be expected to overcome the inhibitory effect of TCDD. However, this was not the case. Taken in combination with the observation that TCDD can exert its antiestrogenic effects at concentrations below that which stimulates appreciable estrogen metabolism (Shiverick and Muther, 1982; DeVito et al., 1992; Liu et al., 1994), these data suggest that an alternative or additional mechanism(s) is involved.

A reduction in ER levels has also been proposed as a mechanism for the decrease in estrogen signaling by TCDD. Gallo and coworkers (DeVito et al., 1992; Tian et al., 1998) demonstrated that TCDD decreased both uterine and hepatic ER and ER mRNA in liver, ovary, and uterus of CD-1 mice. In MCF-7 cells, TCDD reportedly decreased nuclear ER protein, but not mRNA, levels (Wang et al., 1993). Although our results reveal a consistent decrease in both ER α protein and mRNA when the cells are maintained in standard medium, growth in ESM for several days resulted in an unexpected and dramatic decrease in both ER α protein and mRNA. This reduction was not further decreased by TCDD treatment. Interestingly, no corresponding decrease in AhR expression or TCDD-induced CYP1A1 expression was observed in cells

grown in ESM. Accordingly, cells maintained for several days in ESM exhibit a 7-fold greater AhR/ER ratio compared with those grown in standard medium. We would expect that if decreased ER α levels were responsible for the decrease in estrogen signaling, then TCDD treatment of cells grown in ESM should produce a greater antiestrogenic effect. However, cells grown in either medium exhibit not only a similar absolute maximum level of luciferase induction, but also the same degree of TCDD-dependent inhibition of that response. This strongly suggests the presence of "spare estrogen receptors" in the cells, which are not required for the maximal response of these cells to estradiol or for inhibition of that response by TCDD. Thus, if only a fraction of the total ER pool (~10%) is actually required for maximal estrogen signaling, then one can imagine that an observed decrease of 30 to 60% of this pool might have little, if any, effect on estrogen signaling within the cell.

Although measurement of ER levels in the cell is valuable, a more realistic assessment of ER function is to examine ER-DNA binding. Recent studies have suggested that the binding of the AhR to a specific DNA binding site, the iDRE, which overlaps the ER-DNA binding site, prevents ER-ERE binding by virtue of steric hindrance (Krishnan et al., 1995; Gillesby et al., 1997). Several lines of evidence imply, however, that this is not the mechanism responsible for the antiestrogenic effect observed in our studies. First, gel retardation analysis demonstrates that the *in vitro* synthesized AhR-Arnt complex does not bind the ERE responsible for luciferase induction in our cells. Second, using nuclear extracts from treated cells, we do not observe a TCDD-induced AhR-ERE interaction. Whether ER-DNA interactions are disrupted in intact cells remains to be determined. Third, the iDRE mechanism of inhibition seems to be a direct one and is consistent with the observed inhibition of estrogen-induced cathepsin-D gene expression in as early as 1 to 2 h after TCDD treatment (Krishnan et al., 1995). In our hands, TCDD-dependent inhibition of luciferase induction does not occur until 7 to 18 h after treatment, a response that would be more consistent with a delayed or secondary effect. Also, the ability of cycloheximide to block the TCDD-dependent decrease in pS2 and luciferase mRNA is consistent with a mechanism of inhibition, in BG-1 cells, that does not involve direct binding of the AhR to an iDRE. Lastly, and perhaps more importantly, if competition between DNA binding sites were occurring, then the increase in the AhR/ER ratio observed in cells grown in ESM would be expected to produce a greater repression of ER-dependent gene expression. However, we observe the same degree of inhibition by TCDD in both media. In addition, the fact that we do not observe a decrease in ER-DNA binding with nuclear extracts from

TABLE 1

Effect of coactivator proteins on the inhibition of estrogen-induced luciferase activity by TCDD in BG-1 cells transiently cotransfected with pGudLuc7ere and selected coactivators

Values represent mean luciferase activity (relative light units per milligram of protein) \pm S.D. of at least triplicate determinations and are expressed as percentage of estradiol. Experiments were performed two to four times with similar results.

Coactivator	Control	1 nM Estradiol	10 nM TCDD	TCDD + Estradiol
Control	3.93 \pm 1.25	100 \pm 28.85	3.97 \pm 1.77	37.51 \pm 8.63
SRC-1	32.67 \pm 8.34	100 \pm 24.19	22.21 \pm 9.01	50.21 \pm 8.94
p300	17.06 \pm 3.40	100 \pm 18.18	17.39 \pm 5.79	68.08 \pm 9.72
GRIP1	21.43 \pm 6.44	100 \pm 33.39	24.96 \pm 5.65	67.26 \pm 11.56
RIP140	10.19 \pm 1.86	100 \pm 5.15	12.54 \pm 3.02	53.33 \pm 4.86
RAC3	25.47 \pm 5.23	100 \pm 12.04	18.27 \pm 3.91	60.98 \pm 7.74

TCDD-treated cells grown in standard medium, even though we observe a decrease in ER, provides further evidence for the presence of spare receptors that are not required for maximal estrogen signaling or for inhibition of that signaling by TCDD. Although ER-DNA binding was not affected by TCDD in our system, it is possible that TCDD could affect ER conformation, perhaps by altering its phosphorylation state, thereby decreasing its transcriptional activity while not affecting its ability to bind DNA. Gel retardation analysis only provides information as to the DNA binding ability of the ER complex.

The molecular events that occur after ER-DNA binding are still not fully understood. Recent studies have demonstrated that binding of the ER to DNA leads to alterations in chromatin structure and recruitment of coactivator proteins that have since been implicated in bridging the DNA-bound receptor with components of the basal transcriptional machinery, thereby enhancing gene transcription. Early experi-

ments that demonstrated that over-expression of one steroid receptor could inhibit or "squench" the transcriptional activation by another receptor (Meyer et al., 1989) implied that nuclear receptors compete for a limited but critical pool of nuclear cofactors. Although over-expression of selected coactivators had no apparent effect on the TCDD-dependent repression of estrogen responsiveness in BG-1 cells, we realize that there are probably other, as-yet-unknown proteins that are required for signaling of either of these receptors. As a greater number of such proteins are identified and become available, their role in the signaling pathways of the ER and AhR, and their possible involvement in the antiestrogenic action of TCDD, can be assessed. Because the AhR/ER ratio increases 7-fold in ESM, with no corresponding increase in the degree of inhibition of estrogen signaling by TCDD, the antiestrogenic effect in BG-1 cells is probably not mediated by direct competition between the AhR and the ER for coactivator proteins common to both signaling pathways. However, alternate mechanisms of coactivator disruption by TCDD are possible.

The above results, combined with our observation that the inhibitory effect of TCDD is not apparent until 7 to 18 h after treatment, suggest that the inhibition is not a primary event (i.e., it requires AhR-dependent gene expression). This hypothesis is supported by the loss of the TCDD-dependent inhibitory effect when protein synthesis is blocked by cycloheximide. Although cycloheximide blocked the inhibition by TCDD of both luciferase and endogenous pS2 mRNA, a small decrease in control pS2 mRNA in the presence of cycloheximide was observed, and may result from the inhibition of synthesis of a transient protein required for maximal pS2 gene expression. However, this factor does not seem to be required for ER function because luciferase mRNA levels were not decreased with cycloheximide treatment. An additional possibility is the TCDD-dependent repression (either a primary or secondary event) of a transcription factor required for estrogen-dependent gene transcription. In this scenario, not only would target gene expression be repressed, but cellular levels of the cofactor itself would gradually decrease as the protein turns over. Thus, it would be expected that extended TCDD pretreatment would result in a greater degree of inhibition of luciferase, compared with simultaneous treatment. However, our studies reveal that this is not the case. Although there are several possible scenarios in which TCDD can affect ER-dependent gene expression, the available data are currently most consistent with a role for a TCDD-induced protein in the inhibition of estrogen signaling in these cells.

In summary, our data suggest a role for a TCDD-induced factor that acts, after DNA binding, to inhibit estrogen-dependent gene expression at the transcriptional or post-transcriptional level. However, post-transcriptional effects on luciferase reporter gene mRNA and protein are unlikely, because we do not observe the same inhibitory effect of TCDD on induced luciferase activity when luciferase reporter constructs, regulated by different responsive elements, are transfected into BG-1 and other cell lines (data not shown). The antiestrogenic effect of TCDD would not require direct competition between the two receptors and would occur independently of any changes in the AhR/ER ratio and any decrease in ER, provided the maximal ER-DNA binding is not disrupted. A model of the proposed mechanism(s) of the

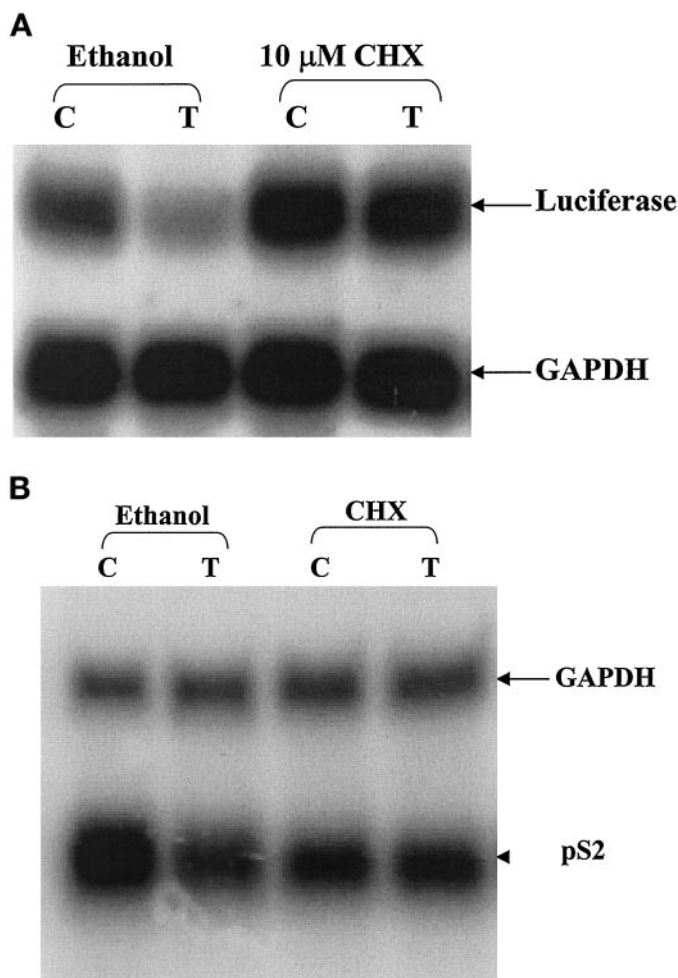


Fig. 8. Cycloheximide blocks TCDD-dependent inhibition of estrogen-induced luciferase and pS2 mRNA expression in BG1Luc4E₂ cells. Cells were grown in standard medium and treated for 24 h with control solvent (C) or 10 nM TCDD (T) with or without 10 μM cycloheximide (CHX). RNA was isolated as described under *Experimental Procedures*. Total RNA (15 μg) was analyzed by Northern blot, and the membranes were probed with radiolabeled luciferase cDNA (A) or radiolabeled pS2 cDNA (B) fragments, and the bands were quantified using a Molecular Dynamics PhosphorImager. Luciferase mRNA and pS2 mRNA values were normalized to GAPDH. These experiments were performed in duplicate (A) or triplicate (B) with similar results, and representative blots are shown.

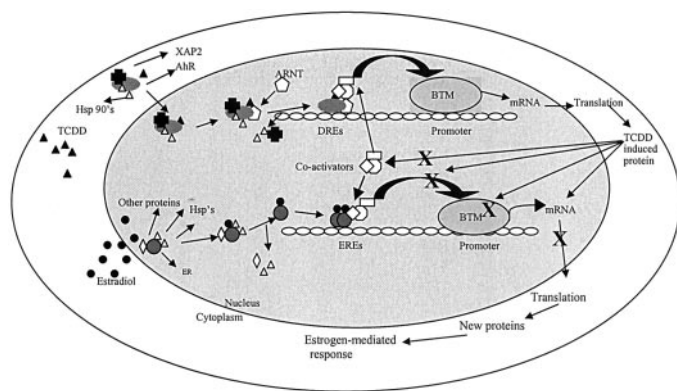


Fig. 9. Mechanism of AhR and ER gene transcription and proposed mechanism for the TCDD-dependent decrease in estrogen signaling in BG1Luc4E₂ cells. BTM, basal transcriptional machinery; XAP2, hepatitis B virus X-associated protein 2.

antiestrogenic action of TCDD in BG-1 cells is shown in Fig. 9. Possible functions of this TCDD-induced "repressor" include an inhibitory action on the estrogen-responsive promoter itself, including inhibition of the ER-preinitiation complex interaction, or disruption of an as yet unidentified ER interacting cofactor. Further studies are under way to identify the nature and target of this unidentified TCDD-induced protein.

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