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Effects of 2,3,7,8-Tetrachlorodibenzo-p-Dioxin, 12-*O*-Tetradecanoylphorbol-13-Acetate and 17β-Estradiol on Estrogen Receptor Regulation in MCF-7 Human Breast Cancer Cells

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Abstract 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) exhibits remarkably potent antiestrogenic activity. To further elucidate the role of estrogen receptor (ER) regulation in this response, we examined the effects of exposure to TCDD in MCF-7 human breast cancer cells on ER mRNA levels by using an RNase protection assay, on ER accumulation by using an ER immunocytochemical essay (ER-ICA), and on ER function by competitive binding assays under conditions of saturating 17β-estradiol (E_2). Comparative studies were conducted with E_2 and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), as both compounds are known to suppress ER expression. Our results indicate that 1 nM E_2 and 100 nM TPA both suppress ER mRNA levels as early as 4 h after exposure and to 33.6% and 16.5% of control levels, respectively, after 72 h. In contrast, no significant effect on ER mRNA levels was attributed to exposure to 10 nM TCDD. A greater than 50% reduction in positive staining was observed by ER-ICA after 72 h exposure to 1 nM E_2 and to 100 nM TPA, while only an 11% reduction in positive staining was observed with 10 nM TCDD. Specific binding of [^{3}H]E_2 under saturating conditions (10 nM E_2) in whole cells was reduced by 50% in cultures exposed to 100 nM TPA, although no effect on binding was observed with exposure to 10 nM TCDD. In contrast, specific binding using subsaturating 1 nM [^{3}H]E_2 was depressed by 49% in MCF-7 cells exposed to 10 nM TCDD for 72 h. This depression was inhibited by a 1-h treatment with 5 μM α-naphthoflavone, which inhibits TCDD-induced, P450-mediated, E_2 metabolism, and subsequent E_2 depletion. In conclusion, while TPA and E_2 effectively down-regulate ER expression, TCDD, under antiestrogenic conditions, has little if any effect on total ER levels in MCF-7 cells, and thus ER modulation is probably not necessary for the suppression of estrogenic activity in MCF-7 cells by TCDD.

Key words: occupied nuclear estrogen receptors, estrogen metabolism, P450, environmental, endocrine-modulating

Environmental exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related halogenated aromatic hydrocarbons continues to evoke public health concerns. TCDD and related compounds induce multiple biological responses in animals and in cultured cells that are species-, strain-, sex-, age-, and target organ-specific. These responses include thymic and lymphoid involution; developmental, reproductive, and hepatic toxicity; dermal lesions; and carcinogenesis [reviewed by Birnbaum, 1994]. Much interest is currently focused on the endocrine-modulating effects of these compounds, particularly with regard to alteration of estrogenic function [Umbreit and Gallo, 1988; Umbreit et al., 1989]. While polychlorinated biphenyls, a related although less potent class of compounds, have been shown to either suppress or enhance estrogenic activity depending on the structures of the individual congeners and their metabolic fate, it is generally agreed that TCDD is an extremely potent antiestrogen [reviewed by Safe et al., 1991].

Evidence of the antiestrogenicity of TCDD is based on the suppression of estrogen enhanced uterotropic responses in rodents [Gallo et al., 1986; Romkes et al., 1987; Umbreit et al., 1988] and on the inhibition of 17β-estradiol(E_2)-stimulated gene expression in animals and in cultures of mammalian derived cells [Gierthy et al., 1987; Safe et al., 1991]. While numerous investigations of the mechanisms involved in the effects caused by

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exposure to TCDD indicate the involvement of aromatic hydrocarbon (Ah) receptor and Ah-receptor-mediated signal transduction, the precise mechanisms of several TCDD-mediated responses, including the antiestrogenic response, remain uncertain. However, it is apparent that many of the biological manifestations of exposure to TCDD are associated with altered differentiation, such as those resulting in teratogenicity [Couture et al., 1990]. It is also recognized that appropriate tissue and organ differentiation depends on unperturbed endocrine function, and it has been proposed that many of the observed biological responses elicited by TCDD can be attributed to alteration of estrogenic action [Umbreit and Gallo, 1988]. Therefore, elucidation of the mechanism of TCDD-mediated antiestrogenicity has been the goal of a number of studies and has led to the generation of several testable hypotheses.

Since TCDD alters the expression of several growth-factor receptors, an attractive hypothesis was that the antiestrogenicity of TCDD is due to the suppression of the estrogen receptor (ER) expression. Studies with rodent liver and uterus generally supported this hypothesis and evidence of suppression of ER expression in response to TCDD exposure was reported [Romkes et al., 1987; Lin et al., 1991; DeVito et al., 1992]. However, subsequent studies with estrogen-responsive MCF-7 human breast cancer cells indicated that TCDD treatment, under conditions which suppressed estrogen-enhanced tissue plasminogen activator and thus demonstrated antiestrogenic potential in this human cell line, had no effect on total cellular ER binding capacity or ligand affinity in estrogen-depleted cultures [Gierthy et al., 1987]. It was concluded from these initial studies that, in the case of the MCF-7 cell system, the antiestrogenicity of TCDD was the result of an Ah-receptor-mediated mechanism that did not involve alteration of ER expression. However, other investigators have reported a decrease in the level of occupied nuclear ER [Harris et al., 1990] and a time-dependent decrease in level of ER mRNA [Lu et al., 1994] in MCF-7 cells treated with TCDD. These subsequent studies have prompted a re-examination of the effects of TCDD on ER expression and function.

In the present study, the effects of exposure to TCDD on the regulation of ER expression and function in MCF-7 cultures were evaluated by using three independent determinations that were chosen to reflect possible changes in ER gene expression or ligand-binding properties of the ER. Levels of ER mRNA were determined by RNase protection assay, ER accumulation was determined by using an ER immunocytochemical assay, and ER binding capacity was determined under conditions of saturating [^{3}H]E_2. The effects of two agents known to alter ER expression, E_2 [Horwitz and McGuire, 1980; Saceda et al., 1988; Cho et al., 1991; Borras et al., 1994] and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) [Ree et al., 1991; Saceda et al., 1991; Tzukerman et al., 1991] were determined in parallel cultures.

MATERIALS AND METHODS

Chemicals

TCDD was obtained from Cambridge Isotope Laboratories, Woburn, MA. Its purity was determined by mass spectroscopy to be >99%. 17β-Estradiol, 12-O-tetradecanoylphorbol-13-acetate, and α-naphthoflavone were obtained from the Sigma Chemical Company, St. Louis, Missouri, DMSO was from the Aldrich Chemical Company, Milwaukee, Wisconsin, and [^{3}H] 17β-estradiol (S.A. = 111 Ci/mmole) was from Dupont NEN Products, Boston, Massachusetts.

Cell Culture

A strain of the MCF-7 cell line, which was originally obtained from Dr. Alberto C. Baldi, Institute of Experimental Biology and Medicine, Buenos Aires, Argentina, was used in all experiments. Cultures were maintained in plastic tissue-culture flasks (T-75) with medium (DC_5) consisting of Dulbecco's modified Eagle's medium (without phenol red) supplemented with 5% bovine calf serum (HyClone, Logan UT) and containing penicillin (100 U/mL), streptomycin (100 μg/mL), insulin (10 ng/mL), L-glutamine (2 mM), and nonessential amino acids (10 mM, from Gibco BRL, Grand Island, NY). The E_2 content of serum lots was determined by radioimmunoassay to be <5 pg/mL [Gierthy et al., 1991], which resulted in <1 pM E_2 in the DC_5 medium. The complete medium was filter-sterilized using 500-mL capacity 0.2-μm pore-size plastic filter units from Nalgene (Rochester, NY) as previously described [Gierthy et al., 1991].

RNase Protection Assay of ER mRNA Levels

The effects of TCDD, E_2, and TPA on the steady-state ER and 36B4 mRNA levels were

determined by using RNase protection with the standard protocol [Ausubel et al., 1987]. The construct for synthesis of the human ER antisense riboprobe (cRNA) was prepared by inserting the *Sma*I-to-*Pvu*II fragment corresponding to positions 244–348 of the human ER cDNA [Greene et al., 1986] into the pBluescript SK- phagemid (Stratagene, La Jolla, CA) at the *Sma*I site. For synthesis of the radiolabeled ER cRNA, the construct was linearized by treatment with *Eco*R1, and synthesis was performed with [α-^{32}P] CTP and T3 polymerase. The construct for synthesis of the 36B4 cRNA consisted of a 220-bp *Pst*I fragment of p36B4 [Masiakowski et al., 1982], which was inserted into the pGEM-3 vector (Promega, Madison WI); the construct was linearized with *Hind*III and probe synthesis was performed with [α-^{32}P]CTP and T7 polymerase. For the analysis of ER and 36B4 mRNA levels in MCF-7 cultures, T-75 flasks at confluence were treated with test compounds or the solvent vehicle, dimethyl sulfoxide (DMSO), at a final concentration of 0.1% v/v in the medium; at the indicated times total cellular RNA was extracted by the method of Chomcynski and Sacchi [1987]. The sequences of the ER and 36B4 mRNAs were protected in the same reactions with 10 μg of MCF-7 cell RNA, assuring that a greater than 20-fold excess of each probe was used. After treatment with RNase A and RNase T1, the protected cRNA fragments were resolved on an 8% acrylamide sequencing gel, which was subjected to autoradiography. The autoradiograms were quantified by scanning densitometry on a Pharmacia LKB ImageMaster densitometer (Pharmacia, Uppsala, Sweden), ensuring that all signals fell within the linear range of absorbance. The relative ER mRNA levels were determined as a ratio of the integrations for the ER band relative to that for the constitutively expressed 36B4 mRNA.

Immunocytochemical Detection of Estrogen Receptor in MCF-7 Cells

Stock MCF-7 cells were seeded into eight-well chamber slides at a concentration of 10^5 cells per 0.4 mL DC_5 per cm^2. After attaining confluence, the cultures were refed with fresh DC_5 or DC_5 containing 0.1% DMSO as solvent control, 10 nM TCDD, 1 nM E_2, or 100 nM TPA. After 72-h exposure, the cultures were fixed and stained with the Abbott ER-ICA Monoclonal Immunocytochemical Assay for the detection of human estrogen receptor using the methods described by the manufacturer. Briefly, after sequential fixation in 3.7% formaldehyde in phosphate-buffered saline (PBS), cold methanol, and cold acetone, the cultures were blocked, treated with the primary monoclonal (rat) antibody (H222) to human ER or control antibody. This was followed by bridging antibody (goat anti-rat IgG) and then rat peroxidase/antiperoxidase complex. The final addition of a chromogen-substrate solution (hydrogen peroxide and diaminobenzidine) produced an insoluble reddish brown product that localizes the ER. The cultures were then counterstained with 0.4% methyl green in sodium acetate buffer, mounted, and evaluated for percent label using a CAS Image Analyzer (Cell Analysis System, Inc., Elmhurst, Ill). Five 40× magnification fields on each of 9 or 18 replicates were evaluated for positive staining using a nuclear threshold setting of 8 and an antibody threshold setting of 18.

[^{3}H]17β-Estradiol Specific Binding Assay

MCF-7 cultures were initiated in 24-well plates (2 cm^2/well) using 2×10^5 cells/mL of DC_5. To determine saturation characteristics of [^{3}H] E_2 binding, confluent untreated cultures were exposed to a 10^{-10}–10^{-8} M concentration series of [^{3}H]E_2 in serum-free medium with or without 200-fold unlabeled E_2 to quantify total binding and nonspecific binding. After 1 h of incubation, the labeling medium was removed, the cultures were washed three times with PBS and incubated for 1 h in 200 μL ethanol to extract the [^{3}H]E_2. A 100-μL aliquot of the ethanol solution was added to liquid-scintillation counting fluid and assessed for radioactivity using a Packard Tricarb liquid-scintillation counter. Specific activity was determined by subtracting the nonspecific binding activity from the total binding activity.

To determine the effect of radiolabeled ligand concentration on TCDD- and TPA-mediated alteration of [^{3}H]E_2 specific binding, confluent cultures were refed with DC_5 medium alone or DC_5 medium containing 0.1% DMSO as a solvent control, 10 nM TCDD, or 100 nM TPA, and incubated for 72 h. Where indicated, α-naphthoflavone was added as a 100 × concentrate to 10 nM TCDD-treated cultures for the last hour of treatment. Specific binding of [^{3}H]E_2 was determined as described above using either 1 nM or 10 nM [^{3}H]E_2 with or without a 200-fold excess

of unlabeled E_2 after adjustments were made for cell culture density based on total DNA measurements using the Hoechst dye procedure described previously [Gierthy et al., 1991].

RESULTS

Effects of TCDD, E_2, and TPA on ER mRNA Levels in MCF-7 Cells

To determine the effects of TCDD, E_2, and TPA on ER mRNA levels in MCF-7 cells, a highly sensitive RNase protection assay was developed similar to that described by Saceda et al. [1991]. Constructs were prepared for synthesis of radiolabeled cRNAs to portions of the ER and constitutively expressed 36B4 mRNAs, and the efficacy with which the cRNAs were protected from digestion with RNase A and RNase T1 by incubation with MCF-7 RNA was determined. The RNA-dependent protection of the 220-bp 36B4 (Fig. 1, lanes 2, 3) and the 104-bp ER (Fig. 1, lanes 4, 5) from digestion was observed. The concentrations of the ^{32}P-labeled cRNA probes that were used in the assays were far in excess (greater than 20-fold) of the concentrations of the ER and 36B4 mRNA concentrations in experimental samples. For analysis of the intact 270-bp 36B4 (Fig. 1, lane 6) and 170-bp ER (Fig. 1, lane 7) cRNAs, both of which contain vector sequences, they were diluted 35- and 12-fold, respectively, from the concentrations used in the protection assay for application to the gel. The low background of the assay allowed the protection of the 36B4 and ER sequences in the same incubation (Fig. 1, lanes 8, 9), allowing for greater precision in the determination of ER mRNA levels relative to those of the 36B4 mRNA. In all subsequent assays protection of the 36B4 and ER mRNA sequences was carried out simultaneously.

Autoradiograms from RNase protection assays for the determination of the effects of 72-h exposure to 10 nM TCDD, 1 nM E_2, and 100 nM TPA on ER mRNA levels are shown in Figure 2. Treatment with 10 nM TCDD had no apparent effect (Fig. 2A), although reduced levels of ER mRNA in response to treatment with 1 nME_2 (Fig. 2B) and 100 nM TPA (Fig. 2C) were visually evident. In additional experiments, exposure to TCDD, E_2 and TPA was carried out for 4, 15, and 24 h, and for each time point autoradio-

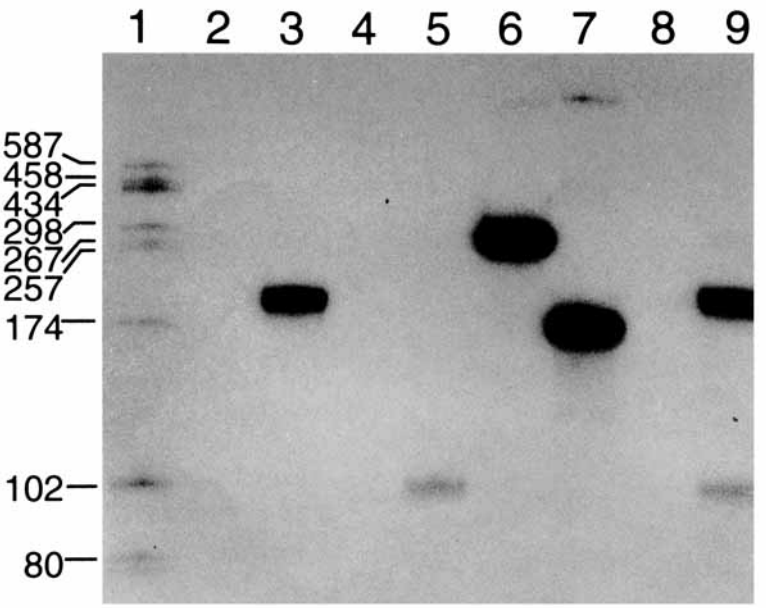

Fig. 1. RNase protection assay of ER and 36B4 mRNAs. Total RNA was isolated from MCF-7 cultures by the method of Chomczynski and Sacchi [1987], and 10-μg samples were incubated with ^{32}P-labeled ER cRNA, 36B4 cRNA, or with both radiolabeled cRNAs for 18 h at 45°C. The samples were then digested with RNase A and RNase T1, and the protected radiolabeled cRNAs were resolved on an 8% sequencing gel. *Lane 1,* DNA size markers (pUC18 digested with HaeIII and labeled with [γ^{32}P]ATP and T4 polynucleotide kinase); *lane 2,* minus RNA control reaction with 36B4 cRNA probe; *lane 3,* protection of the 36B4 sequence with MCF-7 RNA; *lane 4,* minus RNA control reaction with ER cRNA; *lane 5,* protection of the ER sequence with MCF-7 RNA; *lane 6,* intact 36B4 cRNA probe; *lane 7,* Intact ER cRNA probe; *lane 8,* minus RNA control reaction with 36B4 and ER cRNA probes; *lane 9,* protection of the 36B4 and ER sequences with MCF-7 RNA.

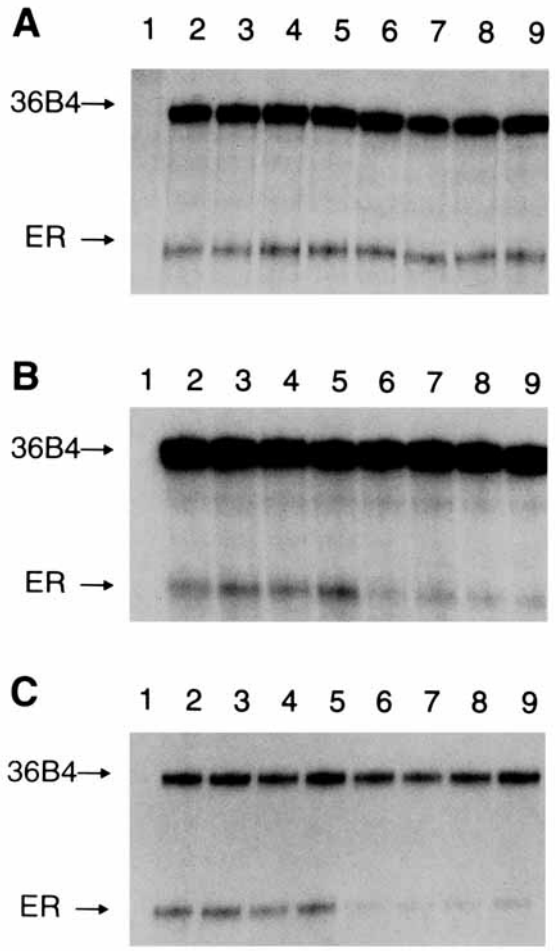

Fig. 2. Effects of TCDD, E_2 and TPA on ER and 36B4 mRNA levels in MCF-7 cells. MCF-7 cultures were treated with 0.1% DMSO or 10 nM TCDD (**A**), 0.1% DMSO or 1 nM E_2 (**B**) and 0.1% DMSO or 100 nM TPA (**C**) for 72 h. RNA was then isolated and analyzed by RNase protection assay. The migration of the protected ^{32}P-labeled ER and 36B4 cRNAs are indicated by arrows. In each panel, *lane 1* is the minus RNA control, *lanes 2–5* are the 0.1% DMSO controls, and *lanes 6–9* are the treatment group.

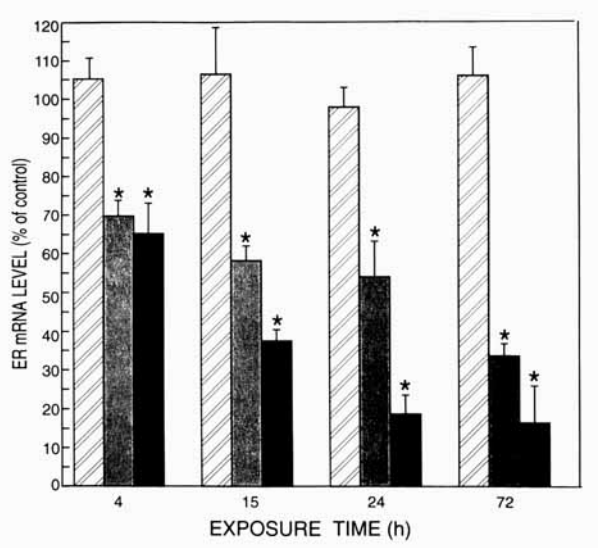

Fig. 3. Time course of the effects of TCDD, E_2, and TPA on ER mRNA levels in MCF-7 cells. Cultures were treated with 0.1% DMSO, 10 nM TCDD (hatched bars), 1 nM E_2 (gray bars), or 100 nM TPA (blackened bars) for the indicated time. At each time point, the ratio of the autoradiographic signals for the ER bands to the 36B4 band were determined and expressed as a percentage of the same ratio obtained for the 0.1% DMSO controls. Mean ±SE for 3 or 4 determinations; *significantly different from control ($P < 0.05$) by the two-tailed t test.

grams were prepared with reduced exposure times to ensure that band intensities fell within the linear range of absorbance by scanning densitometry. The relative ER mRNA levels, determined as the ratio of the ER band integrated absorbance to that of the 36B4 band and expressed as a percentage of the same ratio determined for the 0.1% (v/v) DMSO controls, were shown to be significantly affected in MCF-7 cells by pretreatment with 1 nM E_2 and 100 nM TPA but not with 10 nM TCDD (Fig. 3). After 4 h, the relative ER mRNA levels in cultures exposed to 1 nM E_2 and 100 nM TPA were significantly reduced, to 69.7 ± 4.1 and 65.2 ± 7.9% of control, respectively; after 72 h the relative ER mRNA levels in E_2- and TPA-treated cultures had fallen to 33.6 ± 3.2 and 16.5 ± 9.4% of control. In contrast, the relative ER mRNA levels in cultures treated with 10 nM TCDD were not significantly affected throughout the entire time course.

Effects of TCDD, and E_2, and TPA on Immunochemical Detection of ER in MCF-7 Cells

In previous studies by others [Harris et al., 1990], the suppression of occupied nuclear ER isolated from high-salt nuclear extracts of TCDD-treated MCF-7 cells after a 1-h pretreatment with 1 nM [^3H] E_2 was reported. Since this concentration of E_2 is slightly above the K_d for E_2-ER interaction, it is possible that use of this preloading procedure under conditions of less than ER saturation could lead to equivocal results in regard to estimation of total ER. This is because virtually all of the ER is localized in the nucleus and any metabolism of subsaturating E_2 levels occurring in the cytoplasm would result in a reduction of nuclear E_2 levels and subsequent reduction of occupied nuclear receptor. This would result in an increased loss of the unoccupied ER during the low-salt nuclear preparation. Furthermore,

while the enzyme-linked immunosorbent assay (ELISA) used on the high-salt nuclear extracts in these studies would give an accurate determination of the level of occupied nuclear ER, which is dependent on the condition of the pretreatment, it could give an underestimation of total cellular ER.

Studies were therefore conducted to determine the effect of TCDD on total cellular ER protein by using an ER-specific immunocytochemical assay that was independent of nuclear isolation procedures. The localization of the ER as primarily nuclear, as reported using cytological preparations (Murdoch and Gorski, 1991), was confirmed (Fig. 4). Extensive nuclear staining was observed in both the control (Fig. 4A) and TCDD-treated cultures (Fig. 4B) at approximately equivalent levels, whereas the cultures treated with 1 nM E_2 (Fig. 4C) or 100 nM TPA (Fig. 4D) both showed reduced levels of nuclear staining. Replicate preparations were subjected to image analysis for quantitation of immunoreactive ER by using the CAS system. The total positive staining is presented in Figure 5, which shows, as is consistent with Figures 3 and 4, that treatment with 1 nM E_2 or 100 nM TPA resulted in a large reduction in immunoreactive ER. Conversely, treatment with 10 nM TCDD resulted in a minor, although significant, effect on nuclear staining.

Effect of TCDD on ER Ligand Binding Under Saturating Conditions

Initial studies were performed to determine the saturation kinetics of E_2 binding to the ER of MCF-7 cells. The results shown in Figure 6 indicate that an E_2 concentration of 1 nM approaches saturation, while a concentration of 10 nM is clearly saturating. Further [^{3}H]E_2 binding

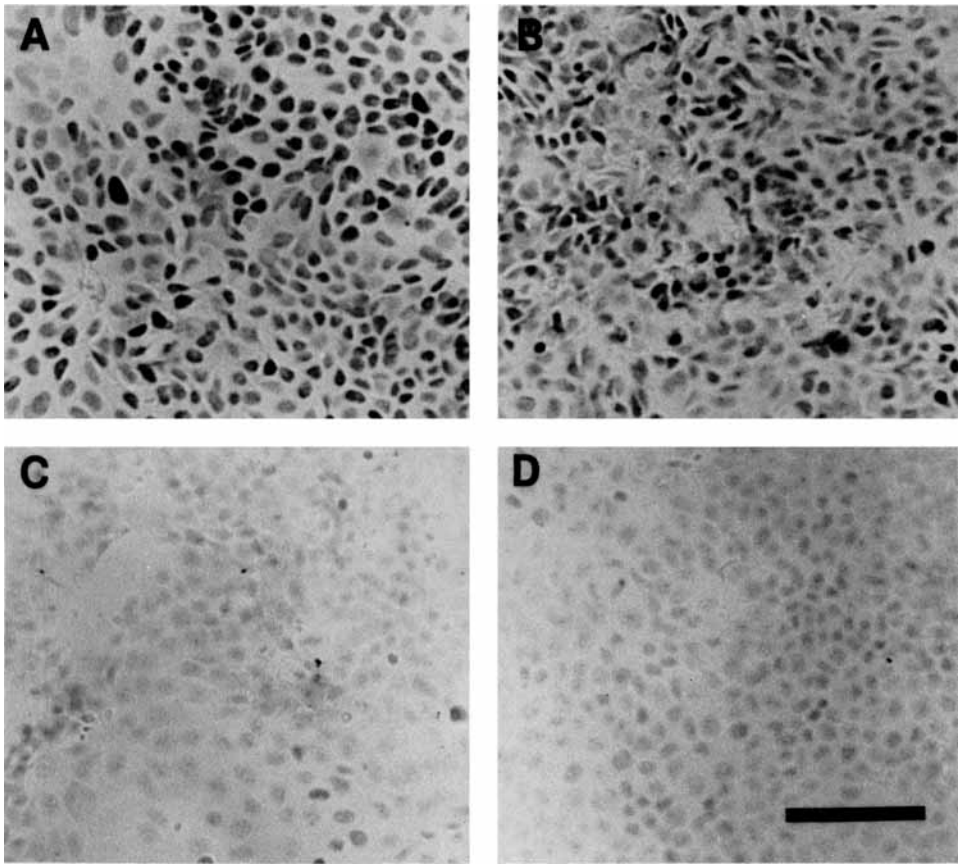

Fig. 4. Effects of TCDD, E_2, and TPA on immunocytochemical detection of ER in MCF-7 cells. Total cellular ER was detected using the Estrogen Receptor-Immunocytochemical Assay (ER-ICA, Abbott). MCF-7 cultures were incubated for 72 h untreated or in the presence of 0.1% DMSO **(A)**, 10 nM TCDD **(B)**, 1 nM E_2 **(C)**, or 100 nM TPA **(D)**. In these representative micrographs, the dark brown DAB reaction product of the enzyme-linked assay is localized to the nuclei and appears as dark gray to black as in panels A and B, while the methyl green counter-stained nuclei appear light gray as in C and D. Untreated and control antibody-treated wells showed no positive DAB staining (data not shown). The bar represents 100 μm.

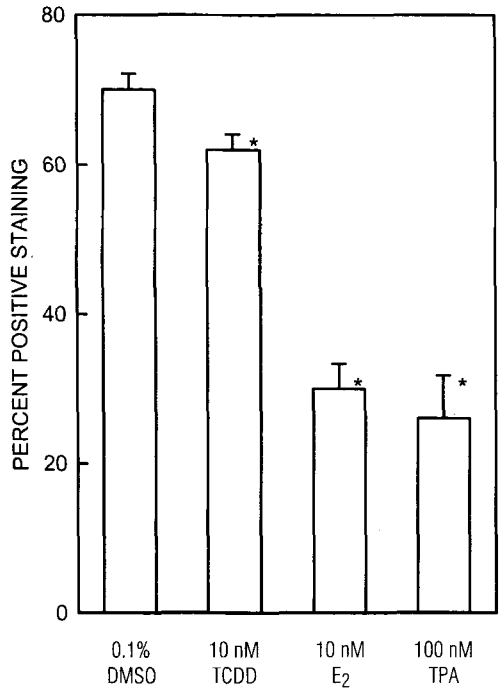

Fig. 5. Quantitation of TCDD, E_2, and TPA treatment on immunocytochemical staining of ER in MCF-7 cells. Total cellular ER was stained using the ER-ICA system as described in figure 4 and percent positive staining was determined using the CAS image analysis system described in Materials and Methods. *$P \leq 0.05$ by Student's *t*-test. For DMSO, n = 18; TCDD, n = 18; E_2, n = 9; TPA n = 9.

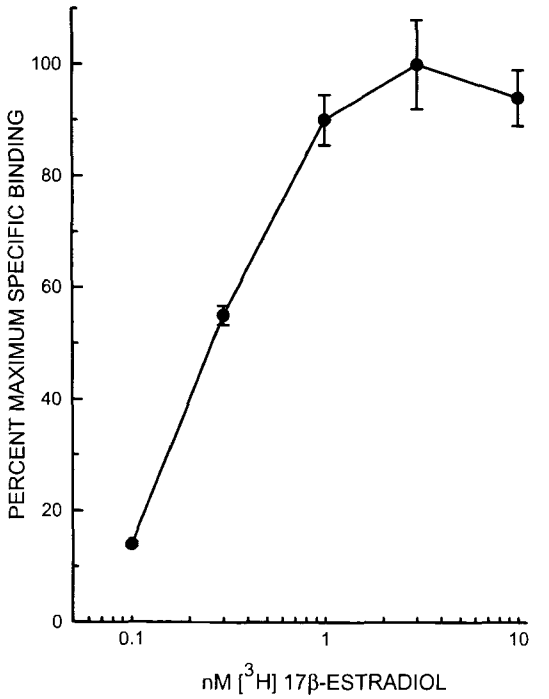

Fig. 6. Saturation kinetics of [^{3}H] 17β-estradiol specific binding in MCF-7 cells. Total [^{3}H]E_2 binding in confluent cultures of intact cells was measured by liquid scintillation counting, using the indicated concentrations in the absence or presence of 200-fold nonlabeled E_2 to determine total binding and nonspecific binding respectively. Specific binding was calculated by subtracting the nonspecific binding from the total binding values and percent maximum specific binding is presented using the value from 3 nM [^{3}H]E_2 as 100%. Each point is the mean of six replicates ±SEM.

studies were performed with MCF-7 cells pretreated for three days with 10 nM TCDD or 100 nM TPA. The results (Fig. 7) indicate that under the less than saturating conditions of 1 nM [^{3}H]E_2, pretreatment with TCDD resulted in a 49% suppression of specifically bound [^{3}H]E_2 as compared with controls. However, this suppression was not observed when the concentration of the [^{3}H]E_2 ligand was increased to 10 nM, a saturating concentration, indicating that the total binding capacity for E_2, or the total receptor number, had not been appreciably altered by pretreatment with TCDD. In contrast, pretreatment with 100 nM TPA caused a 60% decrease in [^{3}H]E_2 binding under the saturating conditions. These results indicate that the down-regulation of ER expression caused by pretreatment with TPA is consistent with a reduction in the receptor number, but suggest that the suppression of ER binding observed with 1 nM [^{3}H]E_2 after pretreatment with TCDD is primarily due to a mechanism unrelated to ER down-regulation.

It is possible that the TCDD-mediated suppression observed with 1 nM [^{3}H]E_2 could be due to availability of less ligand for binding to the nuclear ER. This could be due to metabolism of the [^{3}H]E_2 by TCDD-induced P450s known to catalyze the hydroxylation of E_2 [Spink et al., 1990; 1992a,b; 1994a,b]. Based on the results of co-treatment experiments, it has been proposed that the Ah-receptor ligand α-naphthoflavone (αNF) inhibits the antiestrogenicity of TCDD by competition with TCDD for binding to the Ah receptor [Merchant et al., 1990]. The fact that αNF is also a P450 inhibitor was not considered in this report.

Since αNF both competes with TCDD for binding to the Ah receptor and also inhibits the TCDD-induced metabolism of E_2 catalyzed by CYP 1A1 [Spink et al., 1992a], the mechanism for suppression of TCDD-mediated antiestrogenicity by αNF has not been established. Studies were carried out to clarify this mechanism. Since

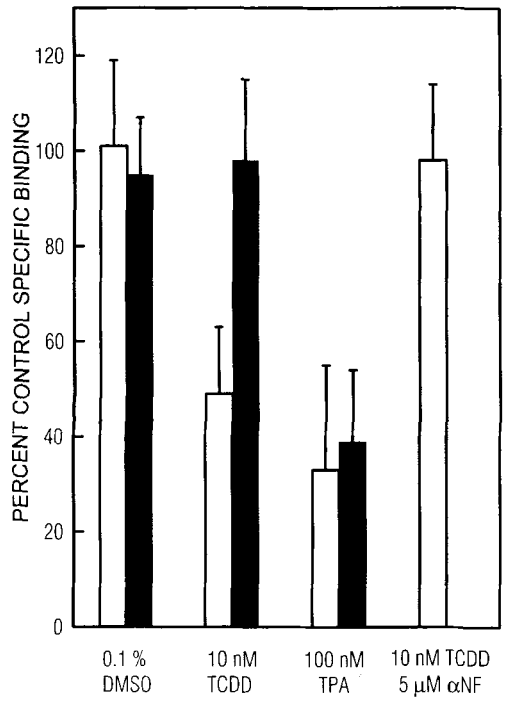

Fig. 7. Effect of TCDD, TPA, and α-naphthoflavone on 1 nM and 10 nM [^{3}H] E_2 specific binding in MCF-7 cells. Percentage of nontreated control specific binding was determined in intact cells using confluent MCF-7 cultures after a 72-h exposure to the 0.1% DMSO vehicle, 10 nM TCDD, and 100 nM TPA together with 1 nM [^{3}H]E_2 (open bars) or 10 nM [^{3}H]E_2 (black bars) as in figure 6. Results of treatment with 5 μM α-naphthoflavone (αNF) during the last hour of the 72-h 10 nM TCDD treatment are shown on the right. Results are a mean of eight replicates ±SEM.

we established that co-incubation of αNF with E_2 for 0.5 h resulted in substantial inhibition of E_2 metabolism under cell free in vitro conditions [Spink et al., 1992a] it was concluded that little time is required for the αNF-P450 interaction to occur. This is in contrast to the time that would be required for the Ah-receptor-mediated effects, which involve inhibition of gene expression and subsequent decay of CYP1A1 activity. Therefore, αNF treatment was undertaken during the last hour of a three-day TCDD treatment before binding assessment using 1 nM [^{3}H]E_2. Induction of P450 by TCDD is known to be maximal by 72 h and is highly persistent, making it unlikely that this late addition of αNF would affect P450 levels, while it would be effective at inhibiting P450 enzyme activity. As shown in Figure 7, this short treatment was sufficient to reduce the TCDD-induced suppression of [^{3}H]E_2 specific binding detected at 1 nM [^{3}H]E_2. This result supports the possibility that TCDD-induced E_2 metabolism through induction of P450 is responsible for the reduction of [^{3}H]E_2 specific binding and reduction of occupied nuclear ER under subsaturating conditions of ligand binding.

DISCUSSION

The present results indicate a lack of TCDD-mediated modulation of total ER in the human estrogen-responsive MCF-7 breast cancer cell line. Exposure to TCDD was shown to have no effect on the ER MRNA levels, little effect on the amount of immunocytochemically detectable ER protein, and no effect on the level of maximal E_2 binding under saturating conditions. These results are consistent with the lack of effect of exposure to TCDD on MCF-7 cell ER-binding affinity and receptor number reported previously from this laboratory using multidose ligand-binding studies with Scatchard analysis [Gierthy et al., 1987]. They do not support mechanisms of ER regulation by TCDD involving alterations in the rate of ER gene transcription, the receptor number, or the total E_2 binding capacity of TCDD-treated MCF-7 cultures.

It has been reported by others that exposure to TCDD results in a down-regulation of the ER in liver [Romkes et al., 1987; Lin et al., 1991; DeVito et al., 1992] and uterus of rodents in vivo. These conclusions were based on ER mRNA steady-state levels determined by Northern analysis, ligand-binding analysis, and by determination of ER protein levels by ELISA. The fact that TCDD down-regulates ER expression in rodent liver and uterus [Romkes et al., 1987; DeVito et al., 1992; Colella and Gallo, 1993] but does not affect ER levels in MCF-7 cells may be explained by well-known differences in the tissue-specific regulation of the ER. In both rodent liver and immature rodent uterus, E_2 up-regulates the ER levels [Shupnick et al., 1989]. In contrast, E_2 is known to down-regulate the ER in the MCF-7 cell line as shown by others [Horwitz and McGuire, 1980; Saceda et al., 1988; Cho et al., 1991; Borras et al., 1994] and confirmed by results presented here (Figs. 2, 3, 5). Thus TCDD would not necessarily be expected to alter expression of the ER in MCF-7 cells in a similar manner as it does in rodent liver and uterus.

It is possible that TCDD may be active through two independent tissue-specific mechanisms for

regulation of ER and antiestrogenic activity, one active in the rodent liver and uterus and another active in the human MCF-7 cell line. However, based on known tissue differences in E_2 regulation of ER, an alternative common mechanism is possible. It is known that TCDD is one of the most potent inducers of P450s in rodents [Keddaris et al., 1991] and in MCF-7 cells [Jaswail et al., 1985; Spink et al., 1990]. Since members of this enzyme superfamily are known to catalyze E_2 metabolism both in rodent tissue [Namkung et al., 1985; Graham et al., 1988] and in MCF-7 cells [Spink et al., 1990; 1992a,b; 1994a,b], possibly the mechanism of TCDD-mediated suppression of the ER in rodent liver and uterus involves intracellular E_2 depletion due to elevated rates of P450 catalyzed metabolism of E_2 to nonestrogenic metabolites. This depletion would probably not alter circulating levels of E_2, owing to a very efficient feedback system that maintains homeostasis. However, tissue-specific metabolism of E_2 and suppression of intracellular levels of the hormone may reduce the concentration of E_2 that reaches the nucleus, where essentially all of the ER is located in association with chromatin [Murdoch and Gorski, 1991]. The depression of intracellular levels of E_2, independent of circulating E_2 levels, could result in the observed down-regulation of E_2-maintained ER levels in liver [DeVito et al., 1992] and the inhibition of E_2-enhanced ER levels in the immature rodent uterus [Romkes et al., 1989]. Specifically, the observed down-regulation of the E_2-regulated expression of ER in vivo in these studies may be a secondary effect of the induction of E_2 metabolism in these tissues by TCDD.

The lack of effect of TCDD on total ER in MCF-7 cells reported here is also consistent with this metabolic intracellular depletion of E_2. Since E_2 exposure has been shown to down-regulate ER expression in this breast cell line [Horwitz and McGuire, 1980; Saceda et al., 1988; Cho et al., 1991; Borras et al., 1994], an environment low in E_2 would result in maximal levels of ER. In this case, TCDD-induced P450 would be expected to have no effect on ER levels, since the system is already low in E_2, and its suppressive effect of ER expression would therefore be absent. However, in an estrogen-rich environment, TCDD-induced metabolism would suppress E_2 levels and result in reduced E_2–ER binding at the nuclear site of the ER, resulting in suppression of E_2-sensitive physiological responses.

Support for this hypothesis would require demonstration of relevant levels of E_2 metabolism induced by corresponding levels of TCDD and verification of a decrease in E_2 reaching the nuclear site of the ER. First, studies carried out in these laboratories showed that TCDD causes a marked increase in the rate of metabolism of E_2 in MCF-7 cells [Spink et al., 1990]. Rates of microsomal P450-catalyzed hydroxylation of E_2 at the C-2, -4, -6α, and -15α positions were each elevated more than tenfold. The TCDD-induced metabolism resulted in the depletion of 10 nM levels of E_2 in MCF-7 cultures within 24 h of E_2 exposure to TCDD-treated cultures. In TCDD-treated cultures 2- and 4-methoxyestradiol, which are without significant estrogenic activity, were the major metabolites formed. In fact, 2-methoxyestradiol has been shown to disrupt cell-cycle events in MCF-7 cells [Lottering et al., 1992] and suppress tumor growth [Fotis et al., 1994]. Lesser amounts of 6α-hydroxyestradiol and 15α-hydroxyestradiol, metabolites with low estrogenic activity, were also formed in response to treatment with TCDD, whereas 16α-hydroxyestradiol, a metabolite with estrogenic activity similar to that of E_2 [Pasqualini et al., 1986], was a very minor metabolite both in microsomal incubations and in assays with intact cells with or without prior exposure to TCDD [Spink et al., 1990, 1992b, 1994a].

The second aspect of supportive evidence for relation of TCDD antiestrogenicity to E_2 depletion through cytoplasmic metabolism is the demonstration of diminished E_2 reaching the nucleus and binding to the ER in TCDD-treated cells. Harris and coworkers [Harris et al., 1990] demonstrated that, under specific conditions, exposing MCF-7 cultures to TCDD results in an approximately 50% suppression of occupied nuclear ER. In these studies there was a 1-h preexposure of intact cells to 1 nM [^{3}H]E_2, which is slightly above the K_d for ER-E_2 interaction and less than the concentration necessary to fully achieve saturation in these cells. Occupied ER levels were then determined after low-salt nuclear isolation followed by sucrose gradient analysis of a high-salt nuclear extract. These procedures ensure recovery of occupied (high-salt extractable) ER [Murdoch and Gorski, 1991]. It should be noted that in these procedures unoccupied ER is not recovered in the nuclear

preparation, and the level of occupied nuclear ER determined is strictly dependent on the nuclear E_2 concentration at the time of extraction. Since ER-ligand binding studies at saturating E_2 show little change in receptor number in MCF-7 cells after exposure to TCDD (Fig. 5), the reduced levels of occupied nuclear ER observed by Harris et al. [1990] with 1 nM E_2 could reflect a reduced nuclear E_2 concentration. Studies using immunological analysis of the high-salt nuclear extracts confirmed this suppression of occupied nuclear ER [Harris et al., 1990]. In addition, a suppressed electrophoretic mobility of a radiolabeled consensus estrogen-response element by incubation with high-salt nuclear extracts from TCDD-treated MCF-7 cells was also cited in support of a decrease in the amount of occupied nuclear ER [Wang et al., 1993].

Since levels of [^{3}H]E_2 that were not saturating were used in the pretreatment before nuclear extraction, there are at least two explanations for the TCDD-mediated suppression of occupied nuclear ER in those studies: (1) there is a transcriptional, translational, or post-translational alteration of ER resulting in decreased receptor number or altered ligand-binding properties; or (2) the decrease in occupied ER in the high-salt nuclear extracts is the result of less [^{3}H]E_2 traversing the cytoplasm and consequently a lower nuclear E_2 concentration. The first possibility is unlikely owing to the results of our previous ligand-binding studies of total ER [Gierthy et al., 1987] and those presented here (Fig. 7).

Studies by Northern analysis showing no effect of TCDD on ER mRNA in MCF-7 cells after 24-h exposure were reported [Wang et al., 1993]; in subsequent studies a 30% reduction in the relative ER mRNA level was observed after 72 h of treatment [Lu et al., 1994]. In the current study, we did not observe any reduction in the relative ER mRNA levels throughout the 72-h time course of treatment with TCDD when compared with concurrent controls treated with only the solvent vehicle (Figs. 2, 3). It should be noted that, in the Northern analyses and in the RNase protection assay used in the current studies, the ER mRNA levels were determined relative to the level of a different constitutive RNA present in MCF-7 cells. The β-tubulin mRNA was used to standardize ER mRNA levels in Northern blot analyses [Wang et al., 1993; Lu et al., 1994], and the 36B4 mRNA was used for standardization in the RNase protection assay reported here as described by Saceda et al. [1991]. Possible minor effects of exposure to TCDD on the β-tubulin and/or 36B4 MRNA levels may account for the fact that we did not observe the slow decrease in the relative ER mRNA level in response to TCDD exposure reported by Lu et al. [1994]. In any event, the effect of exposure to TCDD on ER mRNA levels is, at most, relatively minor and appears to be too slow to account for most of the antiestrogenic effects of TCDD in MCF-7 cells that have been reported.

Other reports and reviews suggest that suppression of occupied nuclear ER in high-salt nuclear extracts of TCDD-treated MCF-7 cells is equivalent to suppression of total nuclear and/or cellular ER [Safe et al., 1991]. In the present report, the effects of TCDD exposure on total (i.e., nuclear) ER levels in MCF-7 cells are clarified and the significance of the TCDD-mediated suppression of occupied-nuclear ER in relation to cytoplasmic E_2 depletion by TCDD-induced metabolism is suggested. Results of the present report confirm and extend the earlier total ER-binding studies by showing that TCDD has little or no effect on ER expression or function in MCF-7 cells as determined by ligand-binding studies performed with saturating levels of [^{3}H]E_2, by RNase protection assays for ER mRNA levels, and by ER immunocytochemical assays.

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