2,3,7,8-Tetrachlorodibenzo-*p*-dioxin causes an extensive alteration of 17 β -estradiol metabolism in MCF-7 breast tumor cells

(antiestrogen/cell foci/aryl hydrocarbon locus)

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ABSTRACT MCF-7 breast tumor cells form multicellular foci *in vitro* when supplemented with 17 β -estradiol (E₂). In the presence of E₂ and the aryl hydrocarbon-receptor agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), MCF-7 cells grow to confluence but do not form foci. To investigate the role of E₂ metabolism in this antiestrogenic effect of TCDD, analyses were performed by capillary GC/MS. The results revealed that pretreatment of MCF-7 cultures with TCDD (10 nM) rapidly depletes E₂. In untreated cultures supplemented with 10 nM E₂, the concentration of free E₂ decreased to 4 nM in the first 12 hr, followed by a slower rate of decline. After 3 days most E₂ in the medium was in conjugated form(s); 1.7 nM was present as free E₂, and 2.9 nM was released by treatment with glucuronidase/sulfatase. In TCDD-treated cultures, E₂ declined to 290 pM in 12 hr and after 2 days was not detected (<100 pM) either as free steroid or after treatment with glucuronidase/sulfatase. Intracellular E₂ and estrone were likewise depleted by pretreatment with TCDD. Microsomes from TCDD-treated cells showed highly elevated aryl hydrocarbon-hydroxylase activity and catalyzed hydroxylations of E₂ at C-2, C-4, C-15 α , and C-6 α with a combined rate of 0.85 nmol/min per nmol of cytochrome P-450 at saturating E₂. These results suggest that depletion of E₂ by enhanced metabolism accounts for the antiestrogenic activity of TCDD in MCF-7 cells.

The estrogen dependence of many breast tumors has been the basis of strategies for the treatment of breast cancer. Historically, ablative procedures such as ovariectomy, adrenalectomy, and hypophysectomy have been employed in treating human breast carcinomas. Currently, antiestrogens are widely used in the adjuvant treatment of the disease. Tamoxifen, which is in clinical use (1), exerts its antiestrogenic effects by antagonism of 17 β -estradiol (E₂) binding to the estrogen receptor. Aromatase inhibitors such as 4-hydroxyandrostenedione are also used in endocrine therapy (2).

Recent studies indicate that antiestrogenic activity is associated with another group of compounds. Certain aromatic hydrocarbons, chlorinated dioxins, and chlorinated dibenzofurans that bind to the aryl hydrocarbon (Ah) receptor exhibit antiestrogenic activity (3-7). The endogenous ligand for the Ah receptor is unknown, and the function of the receptor remains speculative; however, it is established that activation of the Ah locus in mice initiates transcription of several phase I and phase II enzymes, including cytochromes P-450 of the IA class, UDP glucuronosyltransferase, and glutathione transferase (8). A possible mechanism for the antiestrogenic effects of Ah-receptor agonists is that they promote increased metabolism of E₂ by hepatic cytochromes P-450 (4). Alternatively, some investigators suggest that antiestrogenic effects of chlorinated dioxins and diben-

zofurans are independent of cytochrome P-450 induction (3, 5, 7) and are mediated by a downregulation of the estrogen receptor (5, 7).

MCF-7 is a stable cell line derived from a metastatic adenocarcinoma of the human breast that is a widely used model of estrogen-dependent tumors. The growth of MCF-7 cells *in vitro* is stimulated by estrogens and inhibited by antiestrogens. MCF-7 cells form tumors in estrogen-supplemented, immunocompromised rodents (9, 10) and exhibit zones of postconfluent cell accumulation, or foci, *in vitro* (11). These cells express a number of genes in response to estrogens, including those of the progesterone receptor (12), cathepsin D (13), the 24-kDa heat shock protein (14), pS2 (15), and tissue plasminogen activator (16, 17).

The Ah-receptor agonist TCDD showed an antiestrogenic effect in MCF-7 cultures by inhibiting the E₂-regulated expression of tissue plasminogen-activator activity (6). Because MCF-7 cells have TCDD-inducible aryl hydrocarbon hydroxylase (6, 18, 19), a possible mechanism for the antiestrogenic action of TCDD in MCF-7 cultures is that cytochrome P-450 induction results in increased E₂ metabolism. Experiments with ³H-labeled E₂ in the C-2 or C-16 α positions showed increased rates of loss of ³H, indicating an increased hydroxylation at C-2 and, to a lesser extent, at C-16 α in TCDD-treated MCF-7 cultures (20).

While these data support the suggestion that increased hydroxylation of E₂ has a role in the antiestrogenicity of TCDD, they also raise issues concerning the extent of the enhancement of E₂ hydroxylation, the effect on E₂ levels, and the pathways involved. If this activity of TCDD is mediated by increased E₂ metabolism, then the magnitude of the increase must be sufficient to lower E₂ concentrations below physiologically relevant levels. In addition, the metabolites must have low estrogenic activity. To address these issues, GC/MS was used to investigate the effect of TCDD on E₂ metabolism in MCF-7 cells.

METHODS AND MATERIALS

Chemicals. All steroids were from Steraloids (Wilton, NH), except for 15 α -hydroxyestradiol (15 α -OHE₂), which was a gift of Richard Hochberg (Yale University Medical School, New Haven, CT). *N,O*-bis(Trimethylsilyl)-trifluoroacetamide was from Pierce. Deuterium oxide (99.8 atom % ²H), deuterium chloride [20% (wt/vol) in ²H₂O, 99+ atom % ²H], and sodium deuterioxide [40% (wt/vol) in ²H₂O, 99+ atom % ²H] were from Aldrich. Sodium borodeuteride (98 atom % ²H) and type H-2 β -glucuronidase/sulfatase were from Sigma. For use as

Abbreviations: E₁, estrone; E₂, 17 β -estradiol; 2-OHE₂, 2-hydroxyestradiol; 4-OHE₂, 4-hydroxyestradiol; 6 α -OHE₂, 6 α -hydroxyestradiol; 15 α -OHE₂, 15 α -hydroxyestradiol; 16 α -OHE₂, 16 α -hydroxyestradiol; 2-MeOE₂, 2-methoxyestradiol; 4-MeOE₂, 4-methoxyestradiol; Ah, aryl hydrocarbon; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

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internal standards, [16,16,17-²H₃]E₂, 2-OH[1,4,16,16,17-²H₅]E₂, and 16 α -OH[1,3,15,16,17-²H₅]E₂ were synthesized from the corresponding 17-oxosteroids (21). TCDD was obtained from Cambridge Isotope Laboratories (Woburn, MA).

Cell Culture. MCF-7 cells were provided by Alberto C. Baldi (Institute of Experimental Biology and Medicine, Buenos Aires). Cultures were propagated in Dulbecco's modified Eagle's medium supplemented with 5% calf serum and non-essential amino acids and containing 10 ng of insulin, 100 units of penicillin, and 100 μ g of streptomycin per ml. The E₂ concentration in this medium was <1 pM, and phenol red was omitted. Cultures grown in 24-well plates (2 cm² per well), flasks (75 cm²), and roller bottles (1000 cm²) were maintained at 37°C with a humidified air atmosphere containing 5% CO₂. TCDD in dimethyl sulfoxide was applied to preconfluent MCF-7 cultures; untreated cultures received the vehicle alone at 0.1% (vol/vol).

Extraction and Derivatization of Estrogens. Media were buffered at pH 5.0 with 0.1 M sodium acetate and incubated with or without 3000 units of β -glucuronidase and 100 units of sulfatase per ml for 18 hr at 37°C. The internal standard, [²H₃]E₂, was added at 1.6 ng per ml, and 3-ml portions were applied to 1.5 $\times$ 3.5 cm Extrelut QE columns (EM Science). Estrone (E₁) and E₂ were eluted with two 6-ml portions of CH₂Cl₂ (22). Solvent was evaporated under N₂, and 5 μ l of pyridine and 50 μ l of *N,O*-bis(trimethylsilyl)trifluoroacetamide were added. The mixture was heated at 60°C for 30 min, evaporated under N₂, and the residue was redissolved in 4 μ l of benzene for injection on the GC.

For determination of intracellular E₁ and E₂, the cells were harvested, washed with buffered saline, and pelleted by centrifugation. The cells were extracted by sonication in the presence of 10 ml of CH₂Cl₂ containing 8 ng of the internal standard [²H₃]E₂. The CH₂Cl₂ phase was dried over anhydrous Na₂SO₄, evaporated under N₂, and chromatographed on a 0.5 $\times$ 7 cm column of Florasil (Fisher Scientific) with 15% (vol/vol) ethanol in benzene as the solvent (21). The estrogen fraction was collected, evaporated under N₂, and derivatized for analysis.

Microsomal E₂ Hydroxylation. Microsomes from untreated roller-bottle cultures of MCF-7 cells or cultures exposed for 72 hr to medium containing 10 nM TCDD were prepared by differential centrifugation (23). Microsomal incubations (24) contained 50 μ M E₂, 1 mg of microsomal protein, 1.4 mM NADPH, 5 mM MgCl₂, and 2 mM ascorbic acid, and were buffered at pH 7.4 with 0.1 M sodium phosphate in a final volume of 2 ml. On termination of the assay, ascorbic acid was added to 20 mM, 10 ng each of 2-OH[²H₅]E₂ and 16 α -OH[²H₅]E₂ were added as internal standards, and the incubation was extracted three times with 4 ml of ethyl acetate. The organic phase was dried over anhydrous Na₂SO₄, evaporated to dryness under N₂, and derivatized.

Gas Chromatography/Mass Spectrometry. The GC/MS system (Hewlett-Packard) consisted of a model 5890 GC, a model 5970 quadrupole mass analyzer, and a model 9000 computer with MS ChemStation software. The trimethylsilyl ether derivatives of E₂ and its metabolites were resolved on a 0.32 mm (i.d.) $\times$ 30 m fused silica capillary column (SPB-1, Supelco). The carrier gas was helium at a head pressure of 140 kPa. The temperature program consisted of an initial oven temperature of 180°C and a ramp at 4°C per min to the holding temperature of 260°C. The GC injection port and MS interface were maintained at 250°C and 280°C. Electron-impact mass spectra were recorded with 70-eV ionization energy. Selected-ion monitoring of the molecular and major fragment ions of E₂ and its metabolites (25) was performed with a dwell time of 100 msec; 6 to 10 ions were monitored per cycle.

Quantitation of E₂, E₁, and the methoxyestradiols was by peak area at *m/z* 416, *m/z* 342, and *m/z* 446, respectively, relative to the [²H₃]E₂ internal standard at *m/z* 419. The

quantitation of hydroxyestradiols 2-OHE₂, 4-OHE₂, and 15 α -OHE₂ was by peak area at *m/z* 504 relative to the 2-OH[²H₅]E₂ internal standard at *m/z* 509. Because the mass spectrum of the trimethylsilyl derivative of 6 α -OHE₂ does not show an appreciable molecular ion at *m/z* 504, this metabolite was quantitated by peak area at *m/z* 414 relative to the 2-OH[²H₅]E₂ internal standard. Calibration curves for E₂ and each of the metabolites showed good linearity over the 0- to 1-nmol range (*r*² $\geq$ 0.993).

Other Assays. Aryl hydrocarbon hydroxylase activity was measured as described (26). The cytochrome P-450 content of microsomal preparations was determined spectrophotometrically (27). Protein concentrations were determined by the method of Bradford (28).

RESULTS

TCDD Inhibits Estrogen-Dependent Focus Development.

Fig. 1A shows a culture of MCF-7 cells grown for 14 days in the absence of E₂ (<1 pM). The cells formed a confluent monolayer by 5 days, and subsequent cell accumulation was not evident. Replicate cultures continuously exposed to 1 nM E₂ exhibited zonal postconfluent cell accumulation, resulting in focus development beginning at day 9 (Fig. 1B). These areas were multilayer aggregates of cells, the formation of which strictly depended on the presence of an estrogen (J.F.G. and H.W.D., unpublished data). Fig. 1C shows a culture that had been exposed to 1 nM TCDD in addition to 1 nM E₂. Here, the cultures grew to confluence by 5 days, but postconfluent cell accumulation was suppressed, and subsequent focus development was inhibited, resulting in a monolayer similar to that seen when the cultures were grown in the absence of E₂.

TCDD Causes E₂ Depletion in MCF-7 Cultures. In Fig. 2 the concentration of E₂ in media of MCF-7 cultures was monitored for several days after feeding the cultures with media containing 10 nM E₂. The E₂ concentration in control cultures showed an initial rapid decrease over the first 12 hr, followed by a slower rate of decline. Increased amounts of detectable E₂ after treatment of these media with glucuronidase/sulfatase showed that much E₂ was present in conjugated form(s). In TCDD-treated cultures, concentration of free E₂ in the media showed a more rapid decrease, falling to 290 pM in 12 hr and could not be detected (<100 pM) after 28 hr. Furthermore, treatment with glucuronidase/sulfatase failed to release significant E₂ in these media.

Roller-bottle cultures provided a sufficient quantity of cells for the determination of intracellular E₂ and E₁ concentrations. After 24-hr exposure of untreated cells to medium that initially contained 10 nM E₂, intracellular concentrations of E₂ and E₁ were 35 and 13 nM; extracellular concentrations of E₂ and E₁ determined after treatment with glucuronidase/sulfatase were 4.6 and 1.2 nM. These pools represented 62% of the initial E₂. After 24-hr exposure to TCDD-treated cells, intracellular E₂ and E₁ concentrations were 6 and 7 nM, total E₂ in the medium was 260 pM, and E₁ in the medium could not be detected. These pools represented 4% of the initial E₂. After 72-hr exposure, E₁ and E₂ were not detectable in TCDD-treated cultures, either within the cells or in the medium after treatment with glucuronidase/sulfatase.

To further characterize the disappearance of E₂ in the medium of untreated cultures, media were analyzed for methoxyestrogens. Because hydroxylation of E₂ at C-2 occurs in untreated MCF-7 cultures (20, 29) and high levels of catechol *O*-methyltransferase are present in these cells (30, 31), the presence of methoxyestrogens in the medium would be indicative of aromatic hydroxylation. After 72 hr, the methoxyestradiols (MeOE₂) 2-MeOE₂ and 4-MeOE₂ were present in the glucuronidase/sulfatase-treated medium of

FIG. 1. Effect of TCDD on estrogen-dependent focus production in MCF-7 cultures. At 24 hr after seeding, MCF-7 cells were exposed to the normal medium (A), medium containing 1 nM E_2 (B), or medium containing 1 nM E_2 plus 1 nM TCDD (C). Cultures were incubated for 14 days with medium replacement every 4 or 5 days. In B, multicellular foci are evident (arrows) against a monolayer background.

untreated cultures at 1.1 nM and 0.84 nM and in TCDD-treated cultures at 0.65 nM and 0.93 nM.

TCDD Elevates Cytochrome P-450, Aryl Hydrocarbon Hydroxylase, and Microsomal Hydroxylation of E_2 . The total cytochrome P-450 concentrations were 0.014 nmol per mg of protein in microsomes from untreated cells and 0.027 nmol per mg in microsomes from TCDD-treated cells. Aryl hydrocarbon hydroxylase activity was not detected in microsomes from untreated cells (<0.5 pmol/min per mg of protein); the activity in microsomes from TCDD-treated cells was 21.9 ± 6.7 pmol/min per mg of protein (mean $\pm$ SD, $n = 6$).

Microsomes from untreated cells did not produce E_2 metabolites significantly differently from heat-inactivated preparations (Fig. 3A). However, four metabolites were readily identified in incubations with microsomes from

FIG. 2. Time course of E_2 concentration in the media of MCF-7 cultures. Confluent monolayer cultures (75 cm^2) of untreated cells ($\square$, $\circ$) or cells exposed to 10 nM TCDD ($\blacksquare$, $\bullet$) were refed with 5 ml of medium containing 10 nM E_2 at zero time. The media were then collected at the indicated times and analyzed for free E_2 ($\square$, $\blacksquare$) and free plus conjugated E_2 after treatment with glucuronidase/sulfatase ($\circ$, $\bullet$) by GC/MS. (Inset) Selected-ion traces for the molecular ions of the trimethylsilyl ether derivatives of E_2 at m/z 416 (thick line) and the internal standard, $[^2\text{H}_3]E_2$, at m/z 419 (thin line) are shown for the initial medium (A) and for medium after 12-hr exposure to TCDD-treated cells (B).

TCDD-treated cells (Fig. 3B). Peaks with retention times identical to those of the derivatives of 2-OH E_2 and 4-OH E_2

FIG. 3. Hydroxylation of E_2 by microsomes from MCF-7 cells. Incubations with $50\text{ }\mu\text{M}$ E_2 and microsomes from untreated (A) and TCDD-treated (10 nM) (B) MCF-7 cells were extracted and derivatized, and the trimethylsilyl ether derivatives were analyzed by capillary GC/MS with selected-ion monitoring at m/z 504 (thick line) and m/z 414 (thin line). (C) Analysis of an equimolar mixture of E_2 -metabolite standards.

FIG. 4. Electron-impact mass spectra of the trimethylsilyl ether derivatives of 6α -OHE₂ and 15α -OHE₂. (A) The 70-eV mass spectrum of a derivatized metabolite produced in a microsomal incubation of 50 μ M E₂ with microsomes from TCDD-treated MCF-7 cells (upper mass spectrum) is compared with that of synthetic 6α -OHE₂ (inverted lower mass spectrum). (B) The mass spectrum of the derivative of another metabolite produced in the incubation (upper mass spectrum) is compared with that of synthetic 15α -OHE₂ (inverted lower mass spectrum).

were seen, and their identity was confirmed by scanning MS. Two other major peaks were observed with GC retention times (Fig. 3C) and mass spectra identical to those of 6α -OHE₂ and 15α -OHE₂ (Fig. 4).

Microsomes from TCDD-treated cells catalyzed the highest rate of hydroxylation at C-2; lesser rates were observed at C-15 α , C-6 α , and C-4 (Table 1). A small peak was observed at the retention time for 16α -OHE₂ (Fig. 3B), but it was not of sufficient intensity for MS confirmation or quantitation. The total velocity of E₂ hydroxylation at a saturating substrate concentration was 23 pmol/min per mg of protein, or 0.85 nmol/min per nmol of cytochrome P-450, in microsomes from TCDD-treated MCF-7 cells. These hydroxylations depended entirely on the presence of NADPH and were inhibited (46–62%) by gassing the incubation for 60 sec with CO/O₂ (9:1).

DISCUSSION

The antiestrogenic activity of TCDD became apparent when TCDD treatment of immature rodents suppressed E₂-induced increases in uterine wet weights (3, 5). Studies have since shown that TCDD acts as an antiestrogen in MCF-7 cultures by inhibiting the estrogen-regulated expression of tissue plasminogen-activator activity (6) and, here, by inhibiting the estrogen-dependent formation of multicellular foci. Under the same conditions, TCDD treatment resulted in a rapid and near-complete disappearance of E₂, E₁, and conjugates of E₁ and E₂ that are potential sources of active hormone (32, 33) from MCF-7 cultures.

The studies with microsomes indicated that TCDD stimulates hydroxylation of E₂ at C-2, C-4, C-6 α , and C-15 α . The

Table 1. Estradiol hydroxylation by microsomes from MCF-7 cells

Metabolite	Estradiol hydroxylation, pmol/min per mg*	
	Untreated	TCDD-treated
2-OHE ₂	ND	8.4 $\pm$ 0.6
15 α -OHE ₂	ND	6.8 $\pm$ 2.7
6 α -OHE ₂	ND	5.5 $\pm$ 0.9
4-OHE ₂	ND	2.1 $\pm$ 0.2
16 α -OHE ₂	ND	ND
Total activity	—	22.8

ND, not detected (<0.2 pmol/min per mg of protein).

*Velocities were determined in 30-min assays with 50 μ M E₂ (mean $\pm$ SD, *n* = 3).

capacity of the human liver to catalyze hydroxylations of E₂ at all these positions is established (34, 35). Extrahepatic metabolism of E₂ also occurs in the pituitary, ovary, and brain (25, 36). Low levels of E₂ hydroxylase activity at C-2, C-4, and C-16 α were observed in some malignant tumors of the breast (30, 37) and in MCF-7 cells (20, 29, 38). Although in this study E₂ hydroxylase activity was not observed in the short incubations with microsomes from untreated cells, the appearance of 2-MeOE₂ and 4-MeOE₂ in the medium indicates hydroxylation at both C-2 and C-4 with subsequent methylation in untreated cultures. The fact that 2-MeOE₂ and 4-MeOE₂ do not accumulate at higher concentrations in TCDD-treated cultures may be a consequence of multiple hydroxylations of E₂ (39).

The stimulation of metabolism of E₂, its consequent depletion, and conversion to less active metabolites appears to explain the antiestrogenic action of TCDD in MCF-7 cells. The metabolites produced by hydroxylation at C-2 show minimal estrogenic activity (35, 40). Hydroxylation at C-4, however, produces metabolites with some uterotrophic activity. 4-OHE₂ binds with high affinity to estrogen receptors (40, 41), although the affinity is greatly reduced with methylation of the metabolite. In the rat, a reduced, yet substantial, level of uterotrophic activity was seen with 4-MeOE₂ and 4-methoxyestrone (40). 15α -OHE₂ is known to be a nonuterotrophic metabolite, whereas 16α -OHE₂ is an active estrogen in MCF-7 cells (32). However, the C-16 α -hydroxylation pathway is quantitatively much less important than those leading to compounds with low estrogenic activity.

At present it is not known what cytochrome(s) P-450 catalyze the various hydroxylations of E₂ in MCF-7 cells. The marked enhancement of E₂-hydroxylase activity in MCF-7 cells by TCDD suggests the involvement of cytochrome P-450 IA1 and/or P-450 IA2 because they are induced by Ah-receptor agonists (8). Both enzymes from the rat catalyze the hydroxylation of E₂ at C-2 (42, 43); P-450 IA2 also catalyzes hydroxylation at C-4 (43). The hydroxylase activities of P-450 IA1 and P-450 IA2 at the other positions of E₂ were not determined. Because P-450 IA1 is thought to be primarily extrahepatic in humans (44) and is induced by TCDD in MCF-7 cells (8, 18), it would appear at least partially responsible for the increased rate of hydroxylation at C-2.

Stimulation of E₂ metabolism may not be the only antiestrogenic effect of TCDD. A decrease in specific binding of E₂ to the uterine estrogen receptors of TCDD-treated rodents was seen (5, 7). Whether this effect is in some way a consequence of increased E₂ metabolism or whether it is indicative of another interaction between the human analogue of the Ah locus and estrogen-regulated function is not yet known. Recently Vickers *et al.* (45) observed a correlation between the expression of the estrogen receptor and the inducibility of cytochrome P-450 IA1 by TCDD in a wide

series of breast tumor cell lines. The functional basis of the relationship is unknown.

In an investigation of the carcinogenicity of TCDD in rats, Kociba *et al.* (46) observed that, while causing an increased rate of hepatocellular and squamous-cell carcinomas, TCDD treatment resulted in a significantly reduced incidence of tumors in hormonally responsive tissues, including the pituitary, uterus, breast, and adrenals. At TCDD levels that caused slight or no manifestations of toxicity, the overall incidence of tumors was not increased by TCDD. The reduced rates of endometrial (47) and breast cancer (48) in female cigarette smokers may involve activation of the human analogue of the *Ah* locus because cigarette smoke is known to contain a number of *Ah*-receptor agonists (49). Increased hydroxylation of E_2 at C-2 has been observed in female smokers (4).

In summary, treatment of MCF-7 cultures with TCDD inhibits estrogen-dependent focus development, which is characteristic of the tumor origin of these cells and depletes E_2 in this tumor cell line by enhancement of E_2 -hydroxylase activity. These results suggest that estrogen depletion through activation of the human analogue of the human analogue of the *Ah* locus might provide an alternate strategy besides estrogen-receptor blockade by antagonists and blockage of E_2 synthesis by aromatase inhibition in the management of estrogen-dependent breast carcinomas.

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