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The Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin on Estrogen Metabolism in MCF-7 Breast Cancer Cells: Evidence for Induction of a Novel 17β-Estradiol 4-Hydroxylase

David C. Spink,[1,2]* Carrie L. Hayes,[3] Nina R. Young,[1,2] Maro Christou,[4] Thomas R. Sutter,[3] Colin R. Jefcoate[4] and John F. Gierthy[1,2]

[1]Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY 12201-0509, [2]Department of Environmental Health and Toxicology, School of Public Health, University at Albany, State University of New York, Albany, NY 12201-0509, [3]Department of Environmental Health Sciences, School of Hygiene and Public Health, Johns Hopkins University, Baltimore, MD 21205 and [4]Department of Pharmacology, University of Wisconsin Medical School, Madison, WI 53706, U.S.A.

Rates of microsomal 17β-estradiol (E_2) hydroxylation at the C-2, -4, -6α, and -15α positions are each induced greater than 10-fold by treating MCF-7 breast cancer cells with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The TCDD-induced activities at the C-2, -6α and -15α positions have been attributed to cytochrome *P*450 1A1 (CYP1A1); however, the low K_m 4-hydroxylase induced by TCDD appears to be a distinct enzyme. We report here that antibodies to cytochrome *P*450-EF (mouse CYP1B1) selectivity inhibited the C-4 hydroxylation of E_2 catalyzed by microsomes from TCDD-treated MCF-7 cells. Western blots probed with anti-CYP1B antibodies showed the induction of a 52 kDa microsomal protein in response to treatment with TCDD in MCF-7 cells. Western blots of microsomes from HepG2 cells did not show the TCDD-induced 52 kDa protein, and microsomes from TCDD-treated HepG2 cells did not catalyze a low K_m hydroxylation of E_2 at C-4. Cellular metabolism experiments also showed induction of both the C-2 and -4 hydroxylation pathways in TCDD-treated MCF-7 cells as evidenced by elevated 2- and 4-methoxyestradiol ($MeOE_2$) formation. In contrast, TCDD-treated HepG2 cells showed 2-$MeOE_2$ formation predominantly over 4-$MeOE_2$. Northern blots of RNA isolated from untreated and TCDD-treated cells, when probed with the human CYP1B1 cDNA, showed induction of a 5.2 kb RNA in MCF-7 cells but not in HepG2 cells in response to treatment with TCDD. These results provide additional evidence for the induction by TCDD of a novel E_2 4-hydroxylase in MCF-7 cells but not in HepG2 cells and indicate possible endocrine regulatory roles for the newly discovered group of enzymes of the CYP1B subfamily.

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*Correspondence to D. C. Spink at the Wadsworth Center for Laboratories and Research, New York State Department of Health, P.O. Box 509, Albany, NY 12201-0509, U.S.A.

Abbreviations: E_2, 17β-estradiol; E_1, estrone; OHE_2, hydroxyestradiol; $MeOE_2$, methoxyestradiol; TMS, trimethylsilyl; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, GC/MS, gas chromatography/mass spectrometry; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Ah, aromatic hydrocarbon; XRE, xenobiotic responsive element; PAH, polycyclic aromatic hydrocarbon; GADPH, glyceraldehyde 3-phosphate dehydrogenase.

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INTRODUCTION

The metabolism of 17β-estradiol (E_2) in extrahepatic tissues often differs from that in the liver in the relative importance of the 17β-hydroxysteroid dehydrogenase-catalyzed reaction leading to the formation of estrone (E_1), and in the relative rates of hydroxylation at the C-2 and -4 positions, forming the catecholestrogens. In liver, E_2 is predominantly converted to E_1, and the

major sites of hydroxylation are C-2 and -16α, with C-4 hydroxylation being of minor importance [1]. In estrogen-responsive tissues such as breast and uterus the conversion of E_1 to E_2 can be favored [2, 3] and the constitutive rates of E_2 hydroxylation are quite low in comparison to liver. Extrahepatic tissues that have been shown to catalyze significant catecholestrogen formation include the mouse uterus [4], rat anterior pituitary [5], and Syrian hamster kidney [6]. In each of these tissues the relative rate of E_2 4-hydroxylation is comparable to or exceeds that of E_2 2-hydroxylation. Evidence of the existence of specific E_2 4-hydroxylases has been presented [5, 6], although none have been purified or extensively characterized.

In this laboratory we have used MCF-7 breast tumor cells in culture as a model of estrogen-responsive human tissues for studies of the regulation of E_2 metabolism [7, 8]. We found that constitutive rates of E_2 metabolism in untreated cultures were minimal, but treatment with the aromatic hydrocarbon (Ah) receptor agonist 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) caused a marked increase in the rate of E_2 metabolism, resulting in depletion of the hormone from the cultures. Rates of microsomal cytochrome *P*450-catalyzed hydroxylation of E_2 at the C-2, -4, -6α, and -15α positions were each elevated more than 10-fold by treatment with TCDD [7]. Studies with specific antisera and cDNA-expressed enzymes indicated that the hydroxylations at the C-2, C-15α, and C-6α positions of E_2 observed with microsomes from TCDD-treated MCF-7 cells were attributable to the activity of CYP1A1, although the low K_m hydroxylation of E_2 at C-4 appeared to be catalyzed by a distinct TCDD-inducible enzyme [8].

Currently the most thoroughly described cytochrome *P*450s that are induced by Ah-receptor agonists are those of the CYP1 gene family. CYP1A1 and CYP1A2 are induced by TCDD, 3-methylcholanthrene, and other halogenated and nonhalogenated aromatic hydrocarbons. CYP1A1 is induced in liver and in a number of other tissues. The 5′ region of the human *CYP1A1* gene contains four xenobiotic response elements (XREs) that are thought to be the sites of interaction of the ligand-bound Ah receptor, facilitating gene transcription [9]. CYP1A2 appears to be primarily an hepatic enzyme that is expressed constitutively, and the human *CYP1A2* gene contains a single 5′ XRE. Both CYP1A1 and CYP1A2 catalyze C-2 hydroxylation of E_2 at much higher rates than C-4 hydroxylation; this predominance of C-2 hydroxylation over C-4 hydroxylation is observed with CYP1A1 and CYP1A2 from rat [10] and human [8, 11] sources.

Recent studies indicate that Ah-receptor agonists induce the expression of cytochrome *P*450 genes in addition to the two known members of the *CYP1* gene family. Treatment of C3H/10T1/2 mouse embryo fibroblast cells with benz[*a*]anthracene or TCDD induces a polycyclic aromatic hydrocarbon (PAH)-metabolizing enzyme, termed *P*450-EF, that is distinct from CYP1A1 and CYP1A2 [12, 13]. A cytochrome *P*450 that is immunologically related to *P*450-EF has been purified from rat adrenals and has been termed *P*450-RAP [14]. Treatment of the human keratinocyte cell line, SCC-12F, with TCDD induced expression of a number of genes [15], one of which (clone 1) was that of a novel cytochrome *P*450. DNA sequence analysis of this human cDNA clone resulted in the identification of a novel cytochrome *P*450 that is 40% identical to the deduced amino acid sequences of human CYP1A1 and CYP1A2 [16]. This cDNA has been designated as CYP1B1 in accordance with the recommended cytochrome *P*450 nomenclature system [17]; however, the properties and substrate specificity of the cytochrome *P*450 encoded by the human *CYP1B1* gene are entirely unknown. The recent cloning and sequencing of a cDNA of cytochrome *P*450-EF showed that *P*450-EF is 41% identical to mouse CYP1A1 and 81% identical to human CYP1B1, indicating that *P*450-EF is also a member of the CYP1B subfamily and is tentatively referred to as mouse CYP1B1 [18]. The discovery of these novel cytochromes *P*450 that are induced in response to Ah-receptor activation led us to hypothesize that the low K_m E_2 4-hydroxylase activity of TCDD-treated MCF-7 cells is catalyzed by a member of the CYP1B subfamily. In this study we investigated the effects of TCDD on *CYP1B1* gene expression and on E_2 metabolism in two well-characterized cell lines, MCF-7 and HepG2.

MATERIALS AND METHODS

Cell culture

MCF-7 cells originally obtained from Dr Alberto C. Baldi (Institute of Experimental Biology and Medicine, Buenos Aires, Argentina) were used routinely, although in some experiments cells of a MCF-7 strain kindly provided by Dr Nancy E. Davidson (Johns Hopkins University Baltimore, MD) were used. Stock cultures were maintained in plastic tissue culture flasks using medium consisting of Dulbecco's modified Eagle's medium supplemented with 5% bovine calf serum (HyClone, Logan, UT), insulin (10 ng/ml), L-glutamine (2 mM), and nonessential amino acids (10 mM, GIBCO BRL, Grand Island NY). Penicillin (100 U/ml) and streptomycin (100 μg/ml) were also included. The complete medium was filter-sterilized by using 500 ml capacity 0.2 μm pore-size plastic nalgene filter units from Nalgene (Rochester, NY) as previously described [19].

HepG2 cells, obtained from the American Type Culture Collection, were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 μg/ml). Roller-bottle cultures (1000 cm^2) of MCF-7 cells and HepG2 cells were established for the preparation of microsomes. These cultures were either treated with

10 nM TCDD or with the solvent vehicle, 0.1% (v/v) dimethyl sulfoxide (DMSO) in medium, for 72 h prior to preparation of microsomes by the method of Guzelian *et al.* [20]. Cellular E_2 metabolism experiments were carried out with flask cultures (75 cm^2).

E_2 metabolism assays

Rates of E_2 metabolism by both microsomes and cultures of intact cells were determined by using assay procedures employing gas chromatography/mass spectrometry (GC/MS) of the metabolite trimethylsilyl (TMS) derivatives with quantitation by the stable isotope dilution technique [21]. For the determination of microsomal E_2 hydroxylase activity, incubations contained 500 μg of microsomal protein, 1.4 mM NADPH, 5 mM $MgCl_2$, 2 mM ascorbic acid, and varying concentrations of E_2 as substrate. Assays were terminated by extraction with ethyl acetate, and after evaporation of the solvent under N_2, TMS derivatives of the hydroxylated metabolites were prepared as described previously [7].

For the analysis of cellular E_2 metabolism by cultures of MCF-7 and HepG2 cells, cultures were exposed to 10 nM TCDD or the solvent vehicle only (0.1% v/v DMSO in medium) for 72 h. The cultures were then refed with medium containing 100 nM E_2, and after 4 h the medium was recovered for the analysis of E_2 metabolites. Two milliliter aliquots of the medium were adjusted to pH 5 by addition of 10% acetic acid, and type H-2 β-glucuronidase/aryl sulfatase was added (5400 U β-glucuronidase and 130 U aryl sulfatase) followed by incubation at 37°C for 18 h for hydrolysis of the E_2 metabolite conjugates. After hydrolysis of the conjugates, samples were then applied to Extrelut QE columns (EM Science, Cherry Hill, NJ) for solid-phase extraction of the E_2 metabolites. The columns were eluted with two 6-ml portions of methylene chloride. The methylene chloride fraction was evaporated under N_2, and TMS derivatives of the metabolites were prepared for GC/MS analysis.

Analysis of the TMS derivatives of the E_2 metabolites was performed by a slight modification of the GC/MS procedure described previously in that a different capillary GC column was used. Chromatography was performed on a 30 m × 0.2 mm DB-1 column with 0.25-μm film thickness (J & W Scientific, Folsum, CA) with He as carrier gas at a head pressure of 80 kPa. The temperature program for this column consisted of an initial temperature of 180°C and a ramp at 3°C/min to a final temperature of 300°C. Selected-ion monitoring of the molecular and major fragment ions of the E_2 metabolites [21] was performed as described previously [7] with the exception that an additional internal standard was included. For the determination of 2- and 4-$MeOE_2$ by the stable isotope dilution technique, 4,16,16,17-[$^2H_4$]2-$MeOE_2$ was synthesized by the method of Dehennin *et al.* [22] and used as the internal standard. The TMS derivative of this compound was monitored at m/z 450.2. All E_2-metabolite standards were purchased from Steraloids Inc. (Wilton, NH) except 15α-OHE_2, which was a generous gift of Dr Richard Hochberg (Yale University Medical School, New Haven, CT).

Immunoinhibition and immunoblots

The polyclonal antibodies to cytochrome *P*450-EF, IgY, were those raised in female Leghorn White Chickens and purified from their eggs [13]. Polyclonal antibodies to *P*450-RAP were those raised in New Zealand White rabbits [14]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed as described by Laemmli [23], and Western immunoblots of the 7% acrylamide resolving gels were prepared as described previously [24]. Immunoreactive proteins were visualized by using the enhanced chemiluminescence detection system (Amersham Life Science, Bucks., England) according to the protocol of the manufacturer. Immunoinhibition of microsomal E_2 metabolism was determined as described previously [8], with a 20-min preincubation with preimmune or immune IgY followed by a 10-min preincubation with the E_2 substrate, and initiation of the E_2 hydroxylase assay by addition of NADPH.

Isolation of RNA and Northern blots

Total RNA was isolated from MCF-7 and HepG2 cultures by the guanidinium thiocyanate–phenol–chloroform extraction technique of Chomczynski and Sacchi [25]. RNA was denatured by treatment with glyoxal [26] prior to electrophoresis in 1% (w/v) agarose gels. The RNA was transferred to Nytran membranes (Schleicher and Schuell, Keene, NH). The blots were successively probed with CYP1A1 [27] or CYP1B1 (clone 1; Ref. [15]) and glyceraldehyde 3-phosphate dehydrogenase (GADPH) [28] cDNAs that had been ^{32}P-labeled by random priming [29]. Nonspecific hybridization was removed by washing the blots with 1 × SSC and 0.1% SDS for 2 h. The hybridizations were visualized by autoradiography and quantified by densitometry.

Other methods

Protein concentrations were determined by the dye-binding method of Bradford [30] with a commercially available reagent (BioRad, Hercules, CA). Cellular DNA content was determined by using the fluorometric technique with Hoechst dye as described previously [19]. Statistical comparisons were performed by using the two-tailed t-test.

RESULTS

The effects of anti-*P*450-EF antibodies on E_2 hydroxylation catalyzed by microsomes from TCDD-treated MCF-7 cells are shown in Fig. 1. Anti-*P*450-EF IgY caused a selective and concen-

tration-dependent inhibition of E_2 hydroxylation at the C-4 position. The inhibition of hydroxylation at C-4 by anti-*P*450-EF IgY was significant when compared with that by preimmune IgY, whereas the hydroxylation at C-2, -6α, and -15α was not significantly inhibited. This result is the converse of what was observed with anti-*P*450 1A antibodies, where hydroxylation at the C-2, -15α, and -6α positions was inhibited with anti-*P*450 1A IgG, but not hydroxylation at C-4 [8].

The chicken anti-*P*450-EF IgY antibody preparations were previously found to be poor reagents for use in Western immunoblots, despite their potent inhibition of cytochrome *P*450-EF-catalyzed PAH metabolism [13]. However, antibodies raised in rabbits against cytochrome *P*450-RAP were found to be useful for immunodetection in Western blots [14] and also recognized antigenic determinants of cytochrome *P*450-EF [13, 41]. Western immunoblots, when probed with anti-*P*450-RAP antibodies, showed that a protein was induced in MCF-7 cells by treatment with

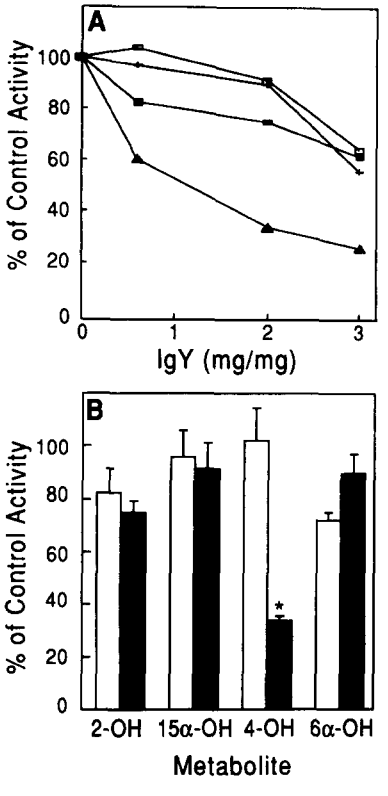

Fig. 1. Effect of anti-*P*450-EF antibodies on E_2 hydroxylation catalyzed by microsomes from TCDD-treated MCF-7 cells. Microsomes were preincubated with antibodies and assayed for hydrolase activity with 25 μM E_2 as described previously [9]. In A, the effect of varying antibody concentration, in mg anti *P*450-EF IgY per mg of microsomal protein, is shown for C-2 (■), C-4 (▲), C-6α (+) and C-15α (□) hydroxylation. In B, the effects of preimmune (□) and anti-P450-EF [17] antibodies (■), each 2 mg IgY per mg microsomal protein, on hydroxylation at each position are shown with mean ± standard error, $n = 4$. The asterisk indicates significant inhibition ($P < 0.05$) for anti-*P*450-EF as compared to preimmune control.

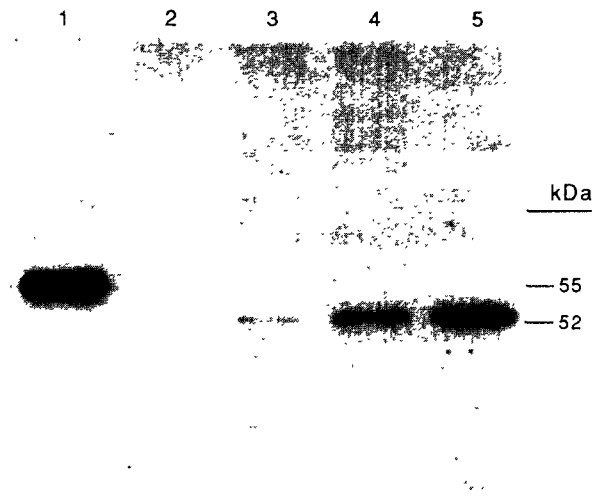

Fig. 2. Western immunoblot of microsomes from MCF-7 cells. Microsomal protein from untreated (lane 2, 6 μg; lane 3, 12 μg) and TCDD-treated (lane 4,6 μg; lane 5, 12 μg) MCF-7 cells was analyzed by SDS–PAGE and Western immunoblot with anti-*P*450-RAP IgG [18]. Immunoreactive proteins were visualized by the enhanced chemiluminescence staining procedure. Microsomal protein (4 μg) from benz[α]anthracene-treated C3H/10T1/2 cells was run in lane 1; the 55 kDa immunoreactive band represents *P*450-EF.

TCDD which was immunoreactive with antibodies to cytochrome *P*450-RAP. A Western immunblot of microsomes from untreated and TCDD-treated MCF-7 cells with immunodetection by using anti-*P*450-RAP antibodies is shown in Fig. 2. The TCDD-induced protein that was immunoreactive with anti-*P*450-EF antibodies migrated in SDS–PAGE with an apparent molecular weight of 52 kDa (lanes 4 and 5), a somewhat greater mobility than cytochrome *P*450-EF from C3H/10T1/2 cells, which migrated with an apparent molecular weight of 55 kDa (lane 1). Western immunoblots of microsomes from HepG2 cells, when probed with anti-*P*450-RAP antibodies, did not show the presence of induction by TCDD of the 52-kDa protein (data not shown). These results are consistent with those of parallel studies in which yet another antibody was used. Western immunoblots probed with rabbit-anti-*P*450-EF showed the induction of a 52-kDa microsomal protein in MCF-7 cells, but not in HepG2 cells, in response to treatment with TCDD [31].

The metabolism of E_2 by microsomes from HepG2 cells was investigated and compared with that which has been characterized for MCF-7 cells [9]. Unlike those from MCF-7 cells, microsomes from untreated (0.1% v/v DMSO control) HepG2 cells catalyzed measurable E_2 hydroxylase activity at the C-2 position (Table 1). Microsomes from TCDD-treated HepG2 cells showed marked induction of E_2 hydroxylase activity at the C-2, -15α, and -6α positions and less induction at C-4. These data were consistent with the known induction of CYP1A1 in HepG2 cells [32]. The

Table 1. E_2 hydroxylation catalyzed by microsomes from untreated and TCDD-treated HepG2 cells

Metabolite	E_2 hydroxylation [pmol/(min·mg)][a]	
	Control	TCDD-treated
2-OHE_2	0.36 ± 0.04	5.95 ± 0.28
6α-OHE_2	ND[b]	5.10 ± 0.24
15α-OHE_2	ND[b]	4.61 ± 0.28
4-OHE_2	ND[b]	0.66 ± 0.07

[a]Velocities were determined with 500 μg of microsomal protein and an E_2 concentration of 25 μM. Values are the mean ± standard error of three determinations.
[b]Not detected [<0.1 pmol/(min·mg)].

C-2, -6α and -15α positions of E_2 are sites of hydroxylation catalyzed by human CYP1A1 [8], which also has minor activity at the C-4 position.

The substrate concentration in the microsomal assay was lowered 50-fold to 0.5 μM E_2 to investigate high-affinity catecholestrogen synthetic activity in HepG2 and MCF-7 microsomes. The low K_m C-4 hydroxylase activity of microsomes from TCDD-treated MCF-7 cells was readily observed under these conditions, as the rate of C-4 hydroxylation was 60% that of C-2 hydroxylation (Table 2). Experiments with microsomes from either control or TCDD-treated HepG2 cells, however, provided no evidence of a low K_m C-4 hydroxylase activity. Hydroxylation of E_2 at C-4 catalyzed by microsomes from TCDD-treated HepG2 cells was barely measurable, as the rate was only 7% that of C-2 hydroxylation. The C-2 hydroxylase activity of microsomes from control HepG2 cultures was observed with 0.5 μM E_2 as substrate, indicating that this is a low K_m hydroxylation.

Since the rapid methylation of 2- and 4-OHE_2 catalyzed by catechol *O*-methyltransferase occurs in many tissues and in both MCF-7 and HepG2 cells, the relative amounts of 2- and 4-$MeOE_2$ released into the medium by intact cells in culture were determined as a measure of the relative rates of the C-2 and C-4 hydroxylation pathways of E_2 metabolism. As both cell lines also have metabolite-conjugating activities, media were treated with β-glucuronidase/aryl sulfatase prior to analysis of 2- and 4-$MeOE_2$ by GC/MS. Although

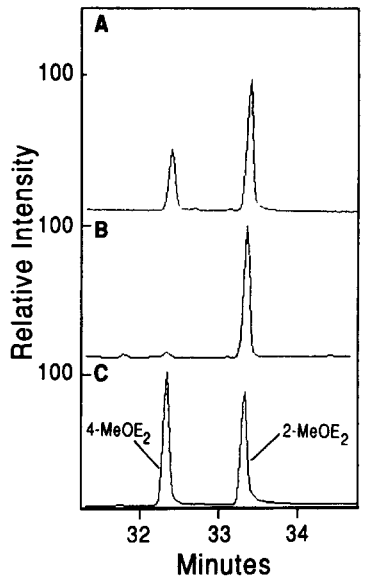

Fig. 3. Methoxyestrogen production by TCDD-treated MCF-7 and HepG2 cells. After treatment for 72 h with 10 nM TCDD, cultures of MCF-7 and HepG2 cells were exposed to medium containing 100 nM E_2. After 4 h the media were removed, treated with glucuronidase/sulfatase for 18 h, and the metabolites were recovered by solid-phase extraction. Trimethylsilyl derivatives of the metabolites were prepared and analyzed by GC/MS. Shown are the selected-ion chromatograms for the molecular ion of the trimethylsilyl derivatives of the methoxyestrogens at *m/z* 446.2 for A, methoxyestrogen production by TCDD-treated MCF-7 cells; for B, methoxy-estrogen production by TCDD-treated HepG2 cultures; and C, 4- and 2-$MeOE_2$ standards, both 19 pmol on column.

the β-glucuronidase/aryl sulfatase used in this study was a crude extract, we found that incubation with this preparation had negligible effect on the recoveries of the two metabolites we measured in the cellular metabolism studies, 2- and 4-$MeOE_2$. When 2- and 4-$MeOE_2$ were added to culture medium followed by incubation with the β-glucuronidase/aryl sulfatase as described, the recoveries of 2- and 4-$MeOE_2$ were 101 ± 4 and 95 ± 2%, respectively (mean ± SD, $n = 4$). Selected-ion chromatograms from the analysis of 2- and 4-$MeOE_2$ in the medium from HepG2 and MCF-7 cultures are shown in Fig. 3. Pretreatment with 10 nM TCDD resulted in 28- and 14-fold increases in the

Table 2. Effect of treatment with TCDD on catecholestrogen formation catalyzed by microsomes from MCF-7 and HepG2 cells

Microsomes		E_2 hydroxylation (pmol/(min·mg))[a]	
		2-OHE_2	4-OHE_2
MCF-7	Control	ND[b]	ND[b]
	10 nM TCDD	0.92 ± 0.05	0.55 ± 0.03
HepG2	Control	0 13 ± 0.01	ND[b]
	10 nM TCDD	0.43 ± 0.02	0.03 ± 0.01

[a]Velocities were determined with 500 μg of microsomal protein and an E_2 concentration of 0.5 μM. Values are mean ± standard error of three determinations.
[b]Not detected [<0.02 pmol/(min·mg)].

Table 3. Effect of treatment with TCDD on methoxyestrogen formation in MCF-7 and HepG2 cells

Cells	Treatment	Methoxyestrogen formation [pmol/(h·mg cell DNA)][a]	
		2-$MeOE_2$	4-$MeOE_2$
MCF-7	Control	1.04 ± 0.09	0.67 ± 0.12
	10 nM TCDD	29.13 ± 0.15[b]	9.34 ± 0.24[b]
HepG2	Control	0 80 ± 0.06	0.17 ± 0.04
	10 nM TCDD	5.42 ± 0.34[b]	0.22 ± 0.01

[a]Results are the mean ± standard error of four cultures with 100 nM E_2 in the medium.
[b]Indicates significantly different ($P < 0.001$) vs controls.

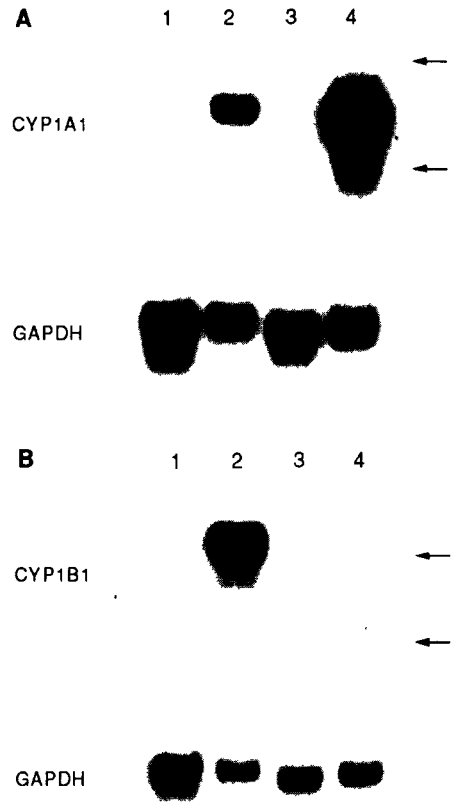

Fig. 4. Northern-blot analysis of RNA from MCF-7 and HepG2 cells. Cultures of MCF-7 and HepG2 cells were treated with medium containing the solvent vehicle (0.1% v/v DMSO) or containing 10 nM TCDD. After 24 h, total RNA was isolated from the cultures control MCF-7 (lane 1), TCDD-treated MCF-7 (lane 2), control HepG2 (lane 3), and TCDD-treated HepG2 (lane 4) by the method of Chomczynski and Sacchi [25] and subjected to Northern analysis as described under Materials and Methods. The blots were successively probed with ^{32}P-labelled CYP1A1 or CYP1B1 and GAPDH cDNAs. In (A) RNA was hybridized with CYP1A1 and GADPH cDNAs; in (B) RNA was hybridized with CYP1B1 and GADPH cDNAs. The arrows to the right of each blot indicate the relative positions of the 28 and 18S ribosomal RNAs.

rates of 2- and 4-MeOE$_2$ production in MCF-7 cells, with the rate of 4-MeOE$_2$ formation 32% that of 2-MeOE$_2$ formation in the TCDD-treated cultures (Table 3). In HepG2 cells, pretreatment with TCDD caused a 6.8-fold increase in the rate of 2-MeOE$_2$ production, whereas production of 4-MeOE$_2$ was not significantly affected.

The microsomal metabolism experiments indicated that TCDD induced an E$_2$4-hydroxylase in MCF-7 cells, but not in HepG2 cells, that was immunologically related to *P*450-EF and *P*450-RAP. We therefore investigated whether induction of an mRNA homologous to that of *P*450-EF could be detected on Northern blots. The cDNA previously referred to as clone 1 [15] that has now been designated CYP1B1 [16] was used to probe Northern blots, as it appears to be the human analog of the recently cloned *P*450-EF cDNA [18]. Shown in Fig. 4 are the results of these Northern blot analyses. Levels of CYP1A1 mRNA were increased 38- and 129-fold after treatment with 10 nM TCDD for 24 h in the MCF-7 [Fig 4(A), lanes 1 and 2] and HepG2 [Fig. 4(A), lanes 3 and 4] cell lines, respectively. In contrast, the expression of the 5.2 kb CYP1B1 mRNA is induced 34-fold in MCF-7 cells [Fig. 4(B), lanes 1 and 2], while its expression is negligible in both controls and TCDD-treated HepG2 cells [Fig. 4(B), lanes 3 and 4].

DISCUSSION

The metabolism of E$_2$ in a number of extrahepatic and hormonally responsive tissues has been investigated extensively, despite the fact that the observed metabolic rates are only a small fraction of those observed in the liver. Metabolism of E$_2$ to the catecholestrogens occurs in a number of extrahepatic tissues including the brain [34], ovaries [21, 35], uterus [4], placenta [36] and kidneys [6]. The physiologic significance of extrahepatic metabolism of E$_2$ is not clear. Rather than simply representing degradation productions of the hormone, autocrine or paracrine roles have been suggested for the catecholestrogens in several tissues including the stimulation of ovarian steroidogenesis [37] and the enabling of implantation [4]. Local synthesis of these highly reactive metabolites would thus be essential for these functions of catecholestrogens. Unlike the liver of both rats and humans, where substantial evidence indicates that cytochromes *P*450 of the CYP3A and, to a lesser extent, CYP1A subfamilies are involved in E$_2$ hydroxylation [10, 11, 33], the relationship of the enzymes mediating extrahepatic catecholestrogen metabolism to the cytochrome *P*450 superfamily is entirely unknown.

In some extrahepatic tissues such as the ovaries of the rat, 2-OHE$_2$ is the principal catecholestrogen formed [35], as it is in rat liver. In contrast, microsomes from the rat anterior pituitary [5] and mouse uterus [4] catalyze low K_m E$_2$ 4-hydroxylation that predominates over the E$_2$ 2-hydroxylase activity. Syrian hamster kidney also appears to express a specific, low K_m E$_2$ 4-hydroxylase, as the microsomal NADPH-dependent E$_2$ 2- and 4-hydroxylases show differential inhibition by fadrozole hydrochloride, a compound that was developed as an inhibitor of aromatase [38], and their expression is affected quite differently by chronic treatment of the hamsters with E$_2$ [6]. To date no purified and reconstituted or cDNA-expressed cytochrome *P*450 has been shown to have the specific, low K_m E$_2$ 4-hydroxylase activity that would account for these observed microsomal activities.

Normal human breast tissue generally has minimal E$_2$ hydroxylase activity, although breast tumors were found to catalyze significant levels of catecholestrogen formation [39]. MCF-7 breast cancer cells catalyze a very low rate of E$_2$ hydroxylation that can be detected

radiometrically and by analysis of methoxyestrogens in the medium. However, exposure of MCF-7 cells to TCDD was found to cause a marked increase in the metabolism of E_2, resulting in depletion of the hormone from the cultures [7]. The rate of cytochrome *P*450-catalyzed hydroxylations of E_2 at the C-2, -15α, -6α and -4 positions were each highly elevated, although kinetic analyses indicated that the C-2 and -4 hydroxylations would be the most physiologically relevant, as these showed more than 7-fold higher catalytic efficiencies (V_{max}/K_m) than the C-15α and -6α, activities [8]. The results of a series of experiments with specific antisera and cDNA-expressed enzymes indicated that the E_2 hydroxylations at C-2, -6α, and -15α positions catalyzed by microsomes from TCDD-treated MCF-7 cells could be attributed to the activities of CYP1A1; however, the low K_m hydroxylation at C-4 appeared to be catalyzed by a different enzyme [8].

The results of the present study indicate that the TCDD-induced E_2 4-hydroxylase in MCF-7 cells is an enzyme that is immunologically distinct from CYP1A1 and is immunoreactive with antibodies to cytochromes *P*450-EF and *P*450-RAP. Cytochrome *P*450-EF is a PAH-metabolizing enzyme that is induced by PAHs and TCDD in C3H/10T1/2 mouse embryo cells [13] and in mouse E041 endometrial cells of stromal origin [40]. Cytochrome *P*450-RAP also catalyzes PAH metabolism, and is expressed in the adrenals, ovaries, and testes of the rat [41]. Antibodies to cytochrome *P*450-EF selectively inhibit the TCDD-induced $E_2$4-hydroxylase activity in microsomes from TCDD-treated MCF-7 cells, and antibodies to cytochromes *P*450-EF and *P*450-RAP recognize a TCDD-induced 52 kDa protein in immunoblots of MCF-7 microsomes. These results suggest that the E_2 4-hydroxylase induced by TCDD in MCF-7 cells is genetically related to cytochromes *P*450-EF and *P*450-RAP, possibly a product of the same gene subfamily.

In mammals, the cytochrome *P*450 superfamily is currently described as consisting of 12 gene families with 22 subfamilies [17]. The physiologic functions of the products of a number of these gene families are clearly defined, such as the roles of *CYP*19 (aromatase) and *CYP*21 (steroid 21-hydroxylase) gene products in the synthesis of steroid hormones and the role of *CYP*7 (cholesterol 7α-hydroxylase) gene products in bile salt formation. The functions of cytochromes *P*450 encoded by genes of the *CYP*1, *CYP*2 and *CYP*3 families are more broadly defined, and appear to be primarily catabolic with substrate specificities not confined to endogenous compounds. Numerous enzymes of these families catalyze the oxidation of drugs and other xenobiotics as well as endogenous steroids. For example, the established enzymes of the *CYP1* family, CYP1A1 and CYP1A2, are often identified by their aryl hydrocarbon hydroxylase and phenacetin *O*-deethylase activities respectively; however, both are also E_2 2-hydroxylases [8, 10, 11].

The identification of cytochrome *P*450-catalyzed PAH metabolism that was induced by Ah-receptor agonists which was not catalyzed by CYP1A1 or CYP1A2 led to the discovery of *P*450-EF [12]. The subsequent identification of *P*450-RAP, which is constitutively expressed in the rat, and the cloning of the human CYP1B1 [15, 16] together with the characterization of *P*450-EF, have provided evidence for additional members of the CYP1 family. The comparison of the sequences of the CYP1B1 [16] and *P*450-EF [18] cDNAs indicate extensive homology; it is likely that *P*450-EF, *P*450-RAP, and CYP1B1 represent the products of orthologous genes or of closely related genes of the rat, mouse and human. The 5.2 kb mRNA that is induced by TCDD in MCF-7 cells hybridizes with the CYP1B1 cDNA under high-stringency conditions, suggesting that the same gene is expressed in MCF-7 and SCC-12F cells [15, 16] in response to treatment with TCDD. Future studies will be directed towards determining whether the low K_m E_2 4-hydroxylase that is induced in MCF-7 cells is a product of the *CYP1B1* gene or of a closely related gene of the cytochrome *P*450 superfamily.

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