Estrogen regulates Ah responsiveness in MCF-7 breast cancer cells

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Introduction

Human cytochrome P450 (CYP)1A1 and CYP1B1 catalyze the metabolic activation of numerous polycyclic aromatic hydrocarbons (PAHs) and ary1 amines to ultimate carcinogens (1–4), and the metabolism of estrogens to catechol estrogens. Human CYP1A1 primarily catalyzes 17β-estradiol (E2) 2-hydroxylation (5), whereas human CYP1B1 primarily catalyzes E2 4-hydroxylation (6,7), a pathway associated with carcinogenesis in experimental animals (8–10), and possibly in humans (11–13). CYP1A1 and CYP1B1 are expressed or are inducible in numerous extrahepatic tissues including breast (14), and CYP1B1 is overexpressed in tumors of several tissues including breast and ovaries (15,16). The potential roles of CYP1A1 and CYP1B1 in carcinogenesis and tumor progression have led to the development of specific inhibitors of CYP1A1 and CYP1B1 as potential anti-mutagenic agents (17,18).

The CYP1A1 and CYP1B1 genes are under the transcriptional control of the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor of the PAS family, which also induces AhR dimerization partners, aryl hydrocarbon receptor nuclear translocator (ARNT) and ARNT2, the AhR repressor, and hypoxia inducible factor 1α (19). Many PAHs are known to induce CYP1A1 and CYP1B1—and thus their own metabolism—through binding to and activation of the AhR. The critical role of the AhR in carcinogenic pathways is evidenced in AhR knockout mice, which are refractory to PAH-induced carcinogenesis (20). While the molecular events constituting the activation of gene transcription by the AhR have been elucidated in considerable detail (21–24), far less is known about the factors that control AhR expression. Results of recent investigations suggest that expression of CYP1A1 and CYP1B1 may be subject to an additional level of control through regulation of AhR expression (25–28).

There is considerable evidence for cross-talk between estrogen- and AhR-regulated responses and signaling. An apparent relationship between estrogen receptor α (ERα) expression and CYP1A1 inducibility in human breast cancer cells has been observed (29–32). However, ERα–AhR cross-talk not only involves CYP1A1/CYP1B1 induction and alteration in E2 metabolism (33), but it may also occur through AhR-mediated inhibitory effects on estrogen-regulated gene expression (34), and possibly by other mechanisms as well (35,36). MCF-7 human breast cancer cells are highly responsive to both estrogens and AhR agonists, and thus provide an ideal system for elucidation of the mechanisms involved in ERα–AhR cross-talk. In this study, we used MCF-7 cells to investigate the effects of short- and long-term presence of estrogen on Ah responsiveness, as evidenced by the constitutive and inducible expression of CYP1A1 and CYP1B1. The results revealed a novel effect of estrogen on AhR expression in MCF-7 cells.

Abbreviations: Ah, aryl hydrocarbon; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; CYP, cytochrome P450; DMEM, Dulbecco’s modified Eagle’s medium; E2, 17β-estradiol; ERα, estrogen receptor α; EROD, ethoxyresorufin O-deethylase; MeOE2, methoxyestradiol; PBS, phosphate-buffered saline; PAH, polycyclic aromatic hydrocarbon; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

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Materials and methods

Cell culture and treatments

The MCF-7 cell line used in these studies was originally obtained from Dr Alberto Baldi (Institute of Experimental Biology and Medicine, Buenos Aires, Argentina) and was maintained at 37°C in a humidified atmosphere with 5% CO2. DF5 medium consisted of Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% (v/v) fetal bovine serum (Hyclone Laboratories, Logan, UT), 10 mM non-essential amino acids, 2 mM L-glutamine, 10 μg/ml insulin, 100 U/ml penicillin and 100 μg/ml streptomycin. DC5 medium differed from DF5 in that it contained 5% bovine calf serum (Cosmic calf serum; Hyclone) rather than fetal bovine serum, and in that phenol red, a compound that elicits estrogen-like activity, was omitted. Stock cultures were maintained and passaged in DF5 or DC5 medium as indicated, but all experimental protocols were performed in DC5 medium.

Assay of cellular E2 metabolism

E2 metabolism assays were performed as described (31,33). Briefly, confluent MCF-7 cultures in 6-well plates with 2 ml medium/well were exposed to 10 nM TCDD or to the solvent vehicle, 0.1% (v/v) DMSO, in DC5 for 72 h, followed by 1 μM E2 in DC5 for 6 h. Media were then recovered, and metabolite conjugates, which are primarily sulfates (37), were hydrolyzed at 37°C for 18 h with β-glucuronidase/sulfatase (Type H-2; Sigma, St Louis, MO). After hydrolysis, media samples were subjected to solid-phase extraction, and trimethylsilyl derivatives of the metabolites were prepared. The metabolite derivatives were analyzed by gas chromatography-mass spectrometry with quantification by stable isotope dilution. Rates of 2- and 4-methoxyestradiol (2- and 4-MeOE2) formation, which were linear for 6 h, were normalized to total protein content as determined by using the bicinchoninic acid protein assay (Pierce, Rockford, IL).

Ethyoxysorfin-O-deethylase (EROD) assay

EROD assays were performed by using a modification of the 96-well plate procedure (38). After treatments, the medium of confluent MCF-7 cultures was replaced with medium containing 4 μM ethoxyresorufin and 10 μM dicumarol (100 μM/well). After 30 min at 37°C, 75 μl of the medium was transferred to a white, opaque 96-well plate (Bioworld Lab Essentials, Dublin, OH). Ethanol (200 μl/well) was added, and the plate was centrifuged at 1500 g for 10 min. Fluorescence was measured in a Fusion Universal Microplate Analyzer (Packard Instrument Co., Meriden, CT) using 535-nm excitation and 590-nm emission filters, and was compared with a standard curve for resorufin prepared in medium and ethanol. Resorufin production was linear for 60 min. The cells were washed twice with phosphate-buffered saline (PBS) before lysis with 0.1% sodium dodecyl sulfate and determination of total protein.

RNA analysis by real-time PCR

Levels of mRNA were quantified by real-time PCR using the LightCycler System (Roche Molecular Biochemicals). After reverse transcription of oligo-dT-primed total RNA with Superscript II (Invitrogen, Carlsbad, CA) and was maintained at 37°C for 10 min and then amplified in cycles consisting of denaturation at 95°C for 5–15 s, annealing at the temperatures indicated in Table I for 5 s, and elongation at 72°C for a time, in seconds, equal to the product length in base pairs divided by 25. At the end of each cycle, fluorescence data were acquired after holding for 5 s at the temperatures indicated in Table I. The sequences of the PCR products were verified by nucleotide sequencing. Each primer set was tested with genomic DNA as the template to verify that no amplification had occurred.

Western immunoblots

Microsomes for the analysis of CYP1A1 and CYP1B1 proteins were prepared as described (33). For analysis of AhR and actin proteins, MCF-7 cells grown in 6-well plates were rinsed twice with PBS and lysed in 0.5 ml/well of 1.1× NuPAGE LDS sample buffer (Invitrogen). These microsomal and cell-lysate samples were subjected to electrophoresis in 10% acrylamide NuPAGE Novex Bis-Tris denaturing gels (Invitrogen) as recommended by the manufacturer. Proteins were blotted onto Immobilon-P polyvinylidene difluoride membranes (Millipore, Bethesda, MD), and blots were blocked in Blotto B (1% bovine serum albumin, 1% non-fat dry milk, 0.05% Tween20 in PBS) for 1 h at room temperature. Blots were then probed with goat polyclonal anti-rat CYP1A1/2 antibody (Daichi, Tokyo, Japan) diluted 1:750 in Blotto B, goat polyclonal anti-human actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:500 in Blotto B, rabbit polyclonal anti-human AhR antibody (Santa Cruz Biotechnology) diluted 1:200 in antibody dilution buffer (2% bovine serum albumin, 5% goat serum, 0.05% Tween20 in PBS) or rabbit polyclonal anti-human CYP1B1 antibody (BD Gentest, Bedford, MA) diluted 1:500 in antibody dilution buffer. Blots were then probed with goat anti-rabbit IgG or bovine anti-goat IgG (Santa Cruz Biotechnology), as appropriate, at 1:20 000 dilution. Immunoreactive proteins were detected by using the West Pico Enhanced Chémiluminescence kit (Pierce, Rockford, IL).

Immunooassay of E2 in sera

E2 was measured using the autoimmunoassay analyzer Elecsys 2010 (Roche Diagnostics Systems, Indianapolis, IN), which employs electrochemiluminescence technology. Samples with or without prior hydrolysis of E2 conjugates by incubation with β-glucuronidase/aryl sulfatase (Type H-2; Sigma) were incubated with E2-specific biotinylated antibody, followed by an additional incubation with the reagent containing streptavidin-coated magnetic micro particles and an E2 derivative–ruthenium complex. The chemiluminescence measured was emitted from the reduced form of ruthenium of the magnetic microparticle-E2–ruthenium complexes that are formed via the link of the streptavidin-biotinylated E2 antibody, with detected signal levels inversely proportional to the E2 concentration.

Transfections and luciferase assays

A CYP1B1-promoter-firefly luciferase construct, p1B1Fluc (44), containing nucleotides –1141 to +5 of the human CYP1B1 promoter (45,46) cloned into pGL3-Basic (Promega, Madison, WI), was transiently co-transfected with the reporter plasmid that contained a 2.7-kb fragment of the CYP1B1 promoter and the renilla luciferase plasmid pRL-TK (Promega). After transfection into MCF-7 cells for 48 h, luciferase activity was measured by using the dual-luciferase reporter assay kit (Promega). The firefly luciferase activity was normalized to renilla luciferase activity as an internal control.
pRL-CMV Renilla luciferase control vector (Promega) into MCF-7 cells by using LipofectAMINE 2000 (Invitrogen). Briefly, 180 ng of the CYP1B1-promoter construct, 20 ng of the pRL-CMV vector, and 300 ng of carrier pBluescript SK vector (Stratagene, La Jolla, CA) in 50 μl of DMEM were combined with 2 μl LipofectAMINE 2000 in 50 μl DMEM. After 30 min, 100 μl of this mixture was added per well to a 24-well plate that contained 500 μl of DC5 medium per well and cells at ~90% confluence. Twenty-four hours after transfection, the medium was removed and the cells were treated with the solvent vehicle, DMSO (0.1 or 0.2% v/v), or 10 nM TCDD as indicated, and were incubated for an additional 48 h. The cells were then assayed for luciferase activities by using the Dual-Luciferase Reporter 1000 Assay System (Promega). A human AhR expression vector, pcDNA3.1(+)AhR, was prepared by subcloning the cDNA of the coding sequence of the human AhR (36) from the pSportAhR2 vector (kindly provided by Dr Chris Bradfield, University of Wisconsin) into the EcoRI/NotI sites of pcDNA3.1(+) (Invitrogen). For co-transfection of this AhR expression vector with the CYP1B1-promoter-luciferase construct, varying levels of pcDNA3.1(+)-AhR were combined with pB1Fluc and pRL-CMV as above, and the amount of carrier plasmid luciferase SK vector DNA included was reduced by the amount of pcDNA3.1(+)AhR added.

Statistical evaluations were performed by using analysis of variance and the t-test, with statistical significance defined as P < 0.05.

Results
Effects of E2 exposure on TCDD-induced E2 metabolism
The activity and inducibility of the 2- and 4-hydroxylation pathways of E2 metabolism were determined by measuring the rates of formation of the terminal stable metabolites, 2- and 4-MeOE2. The effects of E2 on the basal and TCDD-induced E2 metabolism in MCF-7 cells are shown in Figure 1. Exposure to increasing levels of E2 produced opposite effects on the rates of formation of 2- and 4-MeOE2. Addition of E2 to DC5 medium caused a concentration-dependent decrease in the rate of TCDD-induced 2-MeOE2 formation, reflective of a diminishing effect of E2 on the induction of CYP1A1 by TCDD. The rate of formation of 2-MeOE2 was decreased by 35% in the presence of 10 nM E2 and 10 nM TCDD compared with that observed in the presence of TCDD alone. Conversely, E2 caused significant increases in both the basal and TCDD-induced rates of 4-MeOE2 formation, which are reflective of CYP1B1 activity. The basal and TCDD-induced rates of 4-MeOE2 formation were elevated 3.2- and 1.4-fold, respectively, by the addition of 10 nM E2. The ratios of the rates of 4- to 2-MeOE2 formation were significantly increased (P < 0.001) in cultures exposed to E2 alone in comparison with the DMSO controls, and in cultures exposed to E2 plus TCDD in comparison with those receiving TCDD alone.

To investigate whether the effects of E2 on 4-MeOE2 formation were due to changes in CYP1B1 transcriptional activity, we performed transfection experiments with a CYP1B1-promoter firefly luciferase construct. The results shown in Figure 2 indicate effects of E2 on CYP1B1 promoter activity that were very similar to the effects observed for 4-MeOE2 formation. Addition of 10 nM TCDD to the medium caused a 7.6-fold increase in CYP1B1 promoter activity in transfected cells. Addition of E2 caused elevations of both basal and TCDD-induced CYP1B1 promoter activity. At 10 nM E2, basal CYP1B1 promoter activity was elevated 2-fold, while TCDD-induced promoter activity in the presence of 10 nM TCDD was elevated 1.7-fold.

To fully evaluate the effects of E2 on CYP1A1 and CYP1B1 expression it was necessary that we determine the E2 content of the sera used in the media formulations. Immunoassay measurements of E2 content revealed that the fetal bovine serum had a free E2 content of 39 pg/ml and a total E2 content (free plus conjugated forms liberated by treatment with β-glucuronidase/aryl sulfatase) of 1033 pg/ml, whereas the bovine calf serum had a free E2 content of 17 pg/ml and a total E2 content of only 51 pg/ml. These equate to total media E2 concentrations of 0.2 nM in DF5 and 0.009 nM in DC5.

Since MCF-7 cells were shown to respond to TCDD differently with respect to induction of the 2- and 4-hydroxylation pathways of E2 metabolism, dependent on whether E2 was present in the medium, we investigated whether long-term culture in normal (DF5) or low-E2 medium (DC5) affected the AhR-regulated pathways of estrogen metabolism. A comparison of effects of E2 on the TCDD-induced E2 metabolism in cells maintained for 28 passages in DF5 or DC5 is shown in Figure 3. Cells maintained in DC5 medium showed a markedly reduced response to TCDD with respect to the induction of the 2- and 4-hydroxylation pathways of E2 metabolism. The rates of 2- and 4-MeOE2 formation in TCDD-treated cells were reduced by 87 and 77%, respectively, in cells maintained in DC5 compared with those maintained in DF5. When cells that had been maintained long-term in DC5 medium were grown to confluence in DC5 supplemented with 1 nM E2, the robust response to TCDD was not restored; however, addition of E2 had an effect in decreasing the ratio of 2- to 4-MeOE2 formation, similar to that observed for cells maintained in DF5 medium.
Effects of E2 and TCDD on CYP1A1, CYP1B1, AhR, ARNT and ERα mRNA levels

The differences in the effects of TCDD and E2 on E2 metabolism observed in MCF-7 cultures maintained in DC5, compared with those maintained in DF5, suggested differences in gene expression in these cultures. To assess whether (i) the reduced levels of the CYP1A1 and CYP1B1 mRNAs accompanied the reduced rates of E2 metabolism in cultures maintained long-term in DC5 compared with those in DF5 and (ii) whether differing levels of mRNAs encoding AhR, ARNT, ARNT2 and ERα were also observed dependent on the medium in which the cells were maintained, we determined levels of these mRNAs by reverse transcription and real-time PCR with RNA that was isolated from cultures identical to those in which E2 metabolism had been assayed (Figure 3). The results showed significant differences from DMSO control without E2, \( \Delta P < 0.05 \), \( \Delta \Delta P < 0.01 \) and from 10 nM TCDD without E2, \( \Delta \Delta \Delta P < 0.001 \), are indicated.

The levels of ERα mRNA were significantly higher in MCF-7 cells maintained long-term in DC5 than in those in DF5; however, when 1 nM E2 was included in the DC5 medium, reduced levels of ERα mRNA were observed. The levels of the CYP1A1 and CYP1B1 mRNAs closely paralleled the rates of 2- and 4-MeOE2 formation, respectively, determined in identical cultures. Addition of 1 nM E2 to the medium resulted in significantly higher levels of TCDD-induced CYP1B1 mRNA, but also caused a trend toward lower levels of TCDD-induced CYP1A1 mRNA. The levels of CYP1A1 and CYP1B1 mRNA in TCDD-treated cells were reduced by 87 and 79%, respectively, in cells maintained in DC5 compared with those maintained in DF5. Levels of the E2-independent 36B4 mRNA, which encodes a ribosomal protein (39) and is commonly used as a control, showed no significant differences among the treatment groups.

Effects of E2 exposure on TCDD-inducible EROD activity

The EROD assay was used as an additional measure of TCDD-inducible CYP activity. CYP1A1, CYP1A2 and CYP1B1 each have EROD activity, with the order of specific activities being CYP1A1 > CYP1A2 > CYP1B1 (47). When CYP1A1 is present, EROD activity primarily reflects the activity of this enzyme (48). In MCF-7 cultures that had been maintained long-term in DF5 medium, EROD activity was highly
inducible by TCDD (Figure 5). This level of induction, however, was markedly reduced when E2 was present in the medium. In MCF-7 cultures that had been maintained for 30 passages in DC5 medium (DC5) or DC5 medium (DC5) were plated in DC5 medium with or without 1 nM E2 and grown to confluence concurrently with the cultures of Figure 3. After treatment for 72 h with 10 nM TCDD or with the solvent vehicle, 0.1% (v/v) DMSO, with or without 1 nM E2, RNA was isolated, reverse-transcribed, and analyzed by real-time PCR for the ERα, AhR, ARNT, CYP1A1, CYP1B1 and 36B4 mRNAs. Data are presented as amol of target mRNA per μg of total RNA and are the mean ± SE of three independent determinations. Significant differences of key comparisons are indicated, **P < 0.01, ***P < 0.001.

Effects of E2 re-exposure in E2-deprived cells

The observed loss of Ah responsiveness for induction of CYP1A1 and CYP1B1 that accompanied long-term culture
DC5 medium, both the ERα and AhR mRNAs reverted to levels closer to those of MCF-7 cells maintained in DF5. Levels of mRNA encoding CYP1A1 and CYP1B1 showed induction patterns consistent with those of the E2 metabolism experiments (Figure 6), as CYP1A1 and CYP1B1 mRNAs were minimally induced in MCF-7 cells maintained in DC5 compared with those maintained in DF5 or DC5 supplemented with 1 nM E2.

Western immunoblot analyses of the CYP1A1, CYP1B1 and AhR proteins
Analyses by western immunoblot of the CYP1A1, CYP1B1 and AhR proteins in cells maintained in DF5, DC5 and DC5 supplemented with 1 nM E2 revealed similar patterns of expression as were determined for the corresponding mRNA levels, and were consistent with the determinations of E2 metabolism. Inducibility of the CYP1A1 and CYP1B1 proteins was markedly reduced in cultures that were maintained for 32 passages in DC5 compared with those maintained in DF5 (Figure 8). Supplementation of the DC5 medium with 1 nM E2 resulted in a substantial restoration of CYP1A1, CYP1B1 and AhR protein expression. In MCF-7 cultures maintained in DF5, DC5 or DC5 supplemented with 1 nM E2, addition of TCDD to the medium caused a reduction in immunoreactive AhR protein, which has been observed previously with MCF-7 cells (26) and murine Hepa-1c1c7 cells (49).

AhR expression in E2-deprived cells
The reduced AhR mRNA and protein levels observed in cultures maintained in DC5 compared with those maintained in DF5 suggested that the loss of Ah responsiveness in the cells maintained in DC5 was due to inadequate AhR levels. To investigate whether cells maintained long-term in DC5 were deficient in the AhR, we performed an experiment in which varying amounts of an AhR expression vector were transfected, together with a vector containing the CYP1B1-promoter-luciferase reporter, into cells that had been maintained long-term in either DC5 or DF5. When AhR expression was enhanced by co-transfection of the AhR expression vector, stimulation of both basal and TCDD-induced CYP1B1 promoter activity was observed in the cells that were maintained in DC5 (Figure 9). In contrast, there was only a modest stimulation of CYP1B1 promoter activity when the AhR expression vector was co-transfected into cells maintained in DF5.

Fig 6. Effects of E2 re-exposure on TCDD-induced E2 metabolism in E2-deprived MCF-7 cells. MCF-7 cells that had been cultured for 32 passages in DF5 medium (DF5), for 32 passages in DC5 medium (DC5) or for 28 passages in DC5 medium followed by four passages in DC5 medium containing 1 nM E2 (DC5 + E2) were treated for 72 h with 10 nM TCDD or with the solvent vehicle, 0.1% (v/v) DMSO, after which rates of 2- and 4-MeOE2 formation were determined as described in Materials and methods. Data are the mean ± SE of three independent determinations. Significant differences of key comparisons are indicated, ***P < 0.001.

Fig 7. Effects of E2 re-exposure on mRNA levels in E2-deprived MCF-7 cells. MCF-7 cells that had been cultured for 32 passages in DF5 medium (DF5), for 32 passages in DC5 medium (DC5), or for 28 passages in DC5 medium followed by four passages in DC5 medium containing 1 nM E2 (DC5 + E2) were cultured concurrently with those of Figure 5. After treatment for 72 h with 10 nM TCDD or with the solvent vehicle, 0.1% (v/v) DMSO, RNA was isolated, reverse-transcribed, and analyzed by real-time PCR for the ERα, AhR, ARNT, CYP1A1, CYP1B1 and 36B4 mRNAs. Data are presented as amol of target mRNA per µg of total RNA and are the mean ± SE of three independent determinations. Significant differences of key comparisons are indicated, *P < 0.05, **P < 0.01, ***P < 0.001.
Estrogen regulates Ah responsiveness in MCF-7 cells

The MCF-7 line of human breast cancer cells has long served as an important model of estrogen-dependent breast cancer, providing key insights into the regulation of gene expression and cell proliferation by estrogen. Not only have MCF-7 cells been used to characterize short-term effects of estrogen exposure, but it has also been shown by use of estrogen deprivation models that the continued presence of estrogen is required for maintenance of specific gene expression (50–52). We studied both short- and long-term effects of E2 on Ah responsiveness, making use of normal and E2-deprived MCF-7 cultures. The results showed both short- and long-term effects of estrogen on Ah responsiveness with respect to the expression of CYP1A1 and CYP1B1.

Studies of short-term effects of E2 on Ah responsiveness in MCF-7 cells showed an inhibition of the TCDD-mediated induction of CYP1A1 cells by E2 (53–57). Our results are consistent with the reported depression of CYP1A1-catalyzed activities, mRNA levels, and promoter activities in MCF-7 cells by E2 exposure. We extend these findings by showing that, while the TCDD-induced increases in the rates of the E2 2-hydroxylation pathway are lessened by E2, both basal and TCDD-induced rates of E2 4-hydroxylation were elevated by E2 concomitant with the increases in CYP1B1 mRNA and CYP1B1 promoter activity. This change in inducible E2 metabolism may have implications in estrogen carcinogenesis, as the ratio of the rates of 4- to 2-hydroxylation of E2 is considered to be a key determinant (8–13). Although there have been no previous reports showing enhancement of human CYP1B1 expression by estrogen, the basal, but not the benzanthracene-induced expression of CYP1B1 in rat mammary fibroblasts was found to be enhanced by E2 exposure (56).

The molecular basis of the effect of E2 in increasing the CYP1B1 to CYP1A1 expression ratio and the 4- to 2-hydroxylation ratio of AhR-regulated E2 metabolism is not entirely known; however, there is evidence that reduced nuclear factor-1 interactions with the CYP1A1 promoter are involved in diminishing the level of TCDD-induced CYP1A1 mRNA and activity (54). A nuclear factor-1 binding motif present in the proximal promoter region of the CYP1A1 gene was shown to be involved in the regulation of CYP1A1 gene expression (57). Ligand-bound ERα may compete with the activated AhR for nuclear factor-1, resulting in reduced CYP1A1 expression (54). In contrast to that of CYP1A1, the promoter region of the CYP1B1 gene does not contain nuclear factor-1 binding motifs, and there is no evidence that this transcription factor participates in the transcriptional regulation of the CYP1B1 gene. E2 exposure elevated rather than depressed the TCDD-induced CYP1B1 mRNA expression, E2 4-hydroxylation and CYP1B1 promoter activity. The increase in CYP1B1 promoter activity determined with luciferase reporter constructs could be interpreted as evidence of direct transcriptional regulation of the CYP1B1 gene by estrogen; however, the promoter region of the CYP1B1 gene does not contain consensus estrogen-responsive elements (45,46).

The lack of identifiable estrogen-responsive elements in the CYP1B1 promoter may not preclude the regulation of CYP1B1 gene expression by estrogen, since in some cases transcriptional activation by estrogen occurs through interactions of ERα with transcriptional initiation complexes of other transcription factors, including Fos and Jun proteins bound to DNA at AP1 sites (58) and Sp1 proteins bound at Sp1 sites (59). More detailed functional analysis of the CYP1B1 promoter will be required to characterize the observed effect of E2 on CYP1B1 promoter activity. An alternative explanation for the effect of E2 on CYP1B1 expression is that the increases in both basal and TCDD-induced CYP1B1 expression are a consequence of the elevated AhR levels elicited by E2. The basal expression of CYP1B1 is known to require at least one xenobiotic-responsive element (46), suggesting that the constitutive as well as the TCDD-induced expression of CYP1B1 may be regulated by the AhR. Elevated AhR levels in response...
to E2 could therefore enhance CYP1B1 transcription in both the presence and absence of exogenous AhR ligands.

Not only have MCF-7 cells served as a model of short-term effects of estrogen on gene expression, but the adaptive changes of MCF-7 cells to long-term estrogen deprivation have also provided a model relevant to several physiologic states, such as menopause. MCF-7 cells display Model 1 regulation of ERα expression by estrogen (51), meaning that estrogen withdrawal leads to increased ERα expression, and exposure of estrogen-deprived cells to E2 leads to a reduction in ERα expression. Long-term estrogen-deprived MCF-7 cells develop enhanced estrogen sensitivity (52), which appears to be due at least in part to elevated ERα expression. Studies of long-term estrogen deprivation have generally employed sera in which steroids and other lipophilic compounds have been removed by adsorption on charcoal. It cannot be assumed that estrogens or other steroids are the only significant components that are removed by this procedure. An alternative approach that we employed to prepare media with minimal estrogenicity was to use bovine calf serum rather than fetal bovine serum. DC5 prepared with bovine calf serum can be used to investigate the effects of levels of E2 as low as 0.01 nM added to the medium (60). Neither the fetal bovine serum nor the bovine calf serum used in this study showed an appreciable level of free E2. The low estrogenicity of bovine calf serum compared with that of fetal bovine serum is apparently due to the fact that bovine calf serum does not contain the large reservoir of E2 conjugates present in fetal bovine serum. Estrogen conjugates, notably estrogen 3-sulfates, can be hydrolyzed in MCF-7 cells and can elicit estrogenic responses (61). Due to the caveats relating to the differences in the sera, the studies reported here might not be directly comparable with other studies of long-term estrogen-deprived MCF-7 cells. Nonetheless, MCF-7 cells maintained long-term in DC5 medium showed an up-regulation of ERα expression very similar to what has been reported in studies of long-term estrogen deprivation (50,52). In MCF-7 cells maintained long-term in DC5, reduced Ah responsiveness, evidenced by reduced CYP1A1 and CYP1B1 inducibility, accompanied elevated ERα expression. Significantly reduced expression of AhR mRNA in E2-deprived MCF-7 cells suggest that reduced levels of the AhR may be responsible for the diminished Ah responsiveness. The experiments with an AhR expression vector strongly support the contention that AhR is the limiting factor of CYP1A1 and CYP1B1 expression and inducibility in these cells, as enhanced cDNA-directed AhR expression elevated CYP1B1-promoter-driven luciferase activity in the E2-deprived cells. It is intriguing to speculate on an association between increased estrogen sensitivity and reduced Ah responsiveness, since reduced rates of estrogen metabolism catalyzed by CYP1A1 and/or CYP1B1 as well as enhanced ERα expression may contribute to estrogen hypersensitivity.

It is not clear how enhanced Ah responsiveness by estrogen exposure may impact PAH-induced carcinogenesis in various tissues. PAHs induce CYP1 enzymes through AhR activation, and these enzymes in turn catalyze the metabolism of the PAHs. In mouse tumor models, the AhR is required for tumorigenesis (20), yet pre-treatment of animals with the potent and persistent AhR agonist, TCDD, inhibits PAH-induced skin tumorigenesis (62). It should be noted that CYP1 enzymes catalyze the detoxification as well as metabolic activation of PAHs, and the levels of CYP and phase II enzymes within a cell may determine whether a pro-carcinogen is converted to a mutagenic form, or is extensively metabolized to innocuous and readily excreted metabolites.

The regulation of AhR expression is complex, as elevated AhR expression appears to be associated with rapid cell proliferation (63), and AhR expression is subject to cell-specific effects of agents such as transforming growth factor-β1 (28) and phorbol esters (25,26). The up-regulation of AhR expression in PAH-induced mammary carcinogenesis in the rat and AhR over-expression in human pancreatic cancer have been cited as evidence that elevated AhR expression may be a molecular biomarker for the transformation process (64), and that the AhR represents a possible therapeutic target (65). Estrogen causes cell proliferation in the breast and endometrium, and cumulative estrogen exposure is a major risk factor for breast and endometrial cancer. Recent studies showed elevated expression of AhR, but not of ARNT, in the endometrium and myometrium of postmenopausal women receiving continuous estrogen (66). These and our results suggest that, in addition to the stimulation of proliferation of both normal and neoplastic cells by estrogen and the carcinogenic effects of catechol estrogen metabolites, an additional role of estrogen in carcinogenesis may involve the regulation of AhR expression and enhanced Ah responsiveness, leading to elevated inducibility and expression of the pro-carcinogenic bioactivating enzymes, CYP1A1 and CYP1B1.

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