Dioxin and Estrogen Signaling in Lung Adenocarcinoma Cells with Different Aryl Hydrocarbon Receptor/Estrogen Receptor α Phenotypes

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Evidence suggests that estrogen affects the pulmonary response to carcinogenic pollutants, such as dioxins. In this study, we examined dioxin and estrogen signaling cross-talk in lung adenocarcinoma cell lines that were engineered to exhibit different aryl hydrocarbon receptor (AhR)/estrogen receptor (ER) α phenotypes. Data showed that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) weakly antagonized estrogen-activated ER α activity in cells expressing abundant ER α , but little AhR. Increase of AhR expression or presence of a dioxin-responsive element in proximity silenced the antiestrogenic effect of TCDD. AhR was bound to dioxin-responsive element and transcriptionally active in both TCDD-untreated and -treated lung adenocarcinoma cells. 17 β -estradiol (E2) reduced basal and TCDD-induced AhR activity only in ER α -positive cells. AhR and ER α exhibited a protein-protein interaction in the presence of E2. Cotreatment with TCDD moderated this protein interaction. Colocalization of ER α and AhR at the estrogen-responsive site under E2 and TCDD/E2 treatments implied that E2 $\cdot$ ER α might hijack AhR away from the dioxin-responsive site. Increasing the relative expression of AhR to ER α counteracted inhibition of AhR activity by E2 $\cdot$ ER α . When AhR and ER α were both highly expressed, TCDD and E2 up-regulated expression of dual-responsive genes cytochrome P450 (CYP) 1A1 and CYP1B1 in a cumulative manner, increasing the danger of metabolic activation of carcinogens. Whereas TCDD $\cdot$ AhR and E2 $\cdot$ ER α appeared to regulate CYP1B1 separately through their binding sites, E2 $\cdot$ ER α increased the TCDD responsiveness and mRNA expression of CYP1A1 in a noncanonical way. In conclusion, AhR/ER α expression pattern, estrogen level, and promoter context determine the genomic action of dioxin in lung adenocarcinoma cells.

Keywords: estrogen; estrogen receptor α ; dioxin; aryl hydrocarbon receptor; signaling cross-talk

Dioxins and dioxin-like furans and polycyclic aromatic hydrocarbons (PAHs) formed during incomplete combustion have been and continue to be a major threat to lung health. Exposure to dioxins has been found to increase lung cancer death in several industrial cohorts and their respective high-exposure subcohorts. Cumulative dose-response trends for cancer are also seen in low-exposure, long-duration studies (1). Although some dioxin-like PAHs, such as benzo[a]pyrene and anthanthrene, act as a complete carcinogen to induce tumor formation after administration to the lung, dioxins increase tumor incidence only in cases of prior exposure to a known carcinogen in animal carcinogenicity

CLINICAL RELEVANCE

This study demonstrates that hormonal milieu and aryl hydrocarbon receptor (AhR)/estrogen receptor (ER) α phenotype influence the impact of dioxin exposure in lung cells. 17 β -estradiol in association with ER α inhibits AhR trans-activation activity, whereas 2,3,7,8-tetrachlorodibenzo-*p*-dioxin displays antiestrogenicity only under low-AhR circumstances. Increase of relative expression of AhR to ER α counteracts the reciprocal inhibition and facilitates cumulative up-regulation of dual-responsive cytochrome P450 (CYP) 1A1 and CYP1B1 genes by dioxin and estrogen. The latter may increase the risk of cancer due to metabolic carcinogen activation.

tests (2, 3). Dioxins have no direct genotoxic effect, but they may alter the genotoxicity of endogenous and exogenous compounds by increasing metabolic activation catalyzed by cytochrome P450 (CYP) enzymes CYP1A1 and CYP1B1 (1, 2). Indeed, dioxins, as well as dioxin-like compounds, can modulate a spectrum of biological processes, including cell division and differentiation, by activation of a transcription factor, named aryl hydrocarbon receptor (AhR), as a ligand. Ligand-activated AhR heterodimerizes with AhR nuclear translocator, then binds to specific regulatory DNA elements, and induces transcription of target genes (4). Knockout mice confirm that AhR is indispensable for urban air particulate-induced carcinogenesis (5).

Sex influences the pulmonary response to pollutants. When chronically exposed to cigarette smoke, female mice develop emphysema-like pulmonary changes earlier than male mice (6). The lungs of female mice also exhibit higher susceptibility to the cytotoxicity of naphthalene, a PAH commonly present in cigarette smoke and diesel exhaust. The naphthalene-induced lung injuries are correlated to the accumulation of the CYP metabolites of naphthalene (7). Significantly higher levels of CYP1A1 expression and DNA adducts have been detected in female smokers' lungs as compared with male counterparts (8). Estrogen is increasingly recognized as a host factor that predisposes women to lung diseases, including lung cancer. A recent study based on three lung cancer cohorts in the United States and Norway reports a converse relationship between serum estrogen and survival (9). Estrogen may affect men's lung health as well as that of women, because of local production catalyzed by aromatase that converts androgen to estrogen. In patients with non-small-cell lung cancer, aromatase expression and estrogen concentration in carcinoma tissues are frequently higher than in normal lung tissues (10). Like dioxin, estrogen exerts its effects mainly through a ligand-dependent transcription factor, termed estrogen receptor (ER), except that estrogen-activated ER acts as a homodimer (11). The α form of ER increases basal and cigarette smoke-induced CYP1A1 and CYP1B1 expression in human bronchial epithelial cells (12), and has been suggested to be a strong prognostic marker for poor non-small-cell lung cancer survival (9, 13).

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Little is known about the cross-talk between AhR and ER α signaling pathways in lung cells, but it has been intensively investigated in mammary cells. Whereas 17 β -estradiol (E2) exhibited diverse effects, ranging from inhibition to activation, on dioxin-activated AhR activity (14–16), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) inhibited estrogen signaling by a number of different mechanisms in human breast cancer cells. TCDD-activated AhR reduced the estrogen responsiveness of pS2 and cathepsin D by hindering ER α to form an active transcriptional complex at the responsive site through binding to an inhibitory dioxin-responsive element (DRE) in close proximity (17, 18). TCDD also increased proteasome-dependent ER α degradation, particularly with cotreatment with E2. The ER α degradation was paralleled by decreases in E2-stimulated ER α activity and target gene expression (19). Increased dioxin and estrogen metabolism catalyzed by AhR- and ER α -regulated CYP1A1 and CYP1B1 is assumed to impose mutual inhibition on signaling, in addition to autoregulation (20, 21). Inhibitory cross-talk may also arise from competition for shared coactivators, including AhR nuclear translocator, which has been demonstrated to be recruited as an ER α coactivator to an estrogen-responsive promoter independent of AhR (22). Chromatin (Ch) immunoprecipitation (IP) analysis showed that, in the presence of TCDD, AhR recruited ER α to the dioxin-responsive upstream region of CYP1A1 in breast cancer cells. Cotreatment with E2 enhanced the ER α recruitment. Activated AhR might thus take ER α away from ER α target genes (16).

In contrast, 3-methylcholanthrene, a dioxin-like PAH, increased the binding of unliganded ER α , but not E2-bound ER α , to the estrogen-responsive site with help of AhR in MCF-7 breast cancer cells. This AhR-facilitated ER α –DNA interaction increased expression of ER α target genes, pS2 and *c-fos* (23). However, some researchers question the role of AhR in the estrogenic effect of 3-methylcholanthrene, because this compound can activate the transactivation function of ER α directly by serving as a ligand (24, 25).

Current evidence suggests that AhR plays an important role in the carcinogenicity of dioxins and dioxin-like compounds. Activation of AhR may modulate estrogen signaling and *vice versa*. Although lung is a primary target site for airborne toxicants and a secondary site for detoxification of ingested toxicants, our understanding of AhR and ER signaling cross-talk in lung cells is limited. This study was aimed at exploring the signaling activity of AhR and ER α in lung cells with differential AhR/ER α expression. We have focused on the classical genomic actions of AhR and ER α using prototypic ligands, TCDD and E2, respectively, because AhR and ER α exert many of their major biological and toxic effects through the classical pathways. To assess the impact of receptor expression pattern, we constructed several daughter lines of CL1–5 human lung adenocarcinoma cells in which AhR and ER α expression could be induced separately or jointly. Gene regulation in response to dioxin, estrogen, or cotreatment was determined under different AhR/ER α phenotypes and promoter contexts. The influences of estrogen and dioxin on the DNA-binding and protein–protein interaction of AhR and ER α were also examined in this study. The results of this study will provide a foundation for elucidation of the association between AhR/ER α composition and susceptibility to lung cancer.

MATERIALS AND METHODS

Transgenic Cell Line Establishment

AhR and ER α transgenic cell lines were established by stable transfection of tetracycline-on expression plasmids, pTO-AhR/RFP (red fluorescence protein) and pTO-ER α , into human lung adenocarcinoma CL1–5 cells (26) alongside a Tet repressor expression plasmid, pcDNA6/TR (Life Technologies, Grand Island, NY). Cell lines were

Figure 1. Aryl hydrocarbon receptor (AhR), estrogen receptor (ER) α , and β -actin protein expression in CL1–5 and daughter cell lines that were stably transfected with a tetracycline-on AhR or ER α expression system or both. Expression of the AhR and ER α transgenes was highly induced after a 24-hour treatment with 1 μ g/ml doxycycline (Dox).

selected by antibiotic resistance. Expression plasmid construction is described in the online supplement.

Reporter Transfection Analysis

Cells were transiently transfected with pSV40- β gal and one of the luciferase (Luc) reporters: estrogen-responsive element (ERE)-Luc, DRE-Luc, and CYP1B1-Luc, for 8 hours using Lipofectamine 2000 (Life Technologies). Transfected cells were incubated overnight in phenol red-free RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO) plus 3% charcoal/dextran-treated FBS (Life Technologies), followed by a 24-hour treatment with doxycycline (Dox) (1 μ g/ml), E2 (1 nM), and TCDD (10 nM) (Sigma-Aldrich), as indicated. AhR and ER activities were determined in Luc: β gal activity ratios as described previously (27). Reporter construction is described in the online supplement.

Western Blotting and IP–Western Blotting

Protein extraction and Western blotting were performed as described previously (28). Whole-cell extracts (20 μ g/lane) and nuclear extracts (2 μ g/lane) were separated by 10% SDS-PAGE. Rabbit anti-AhR (Enzo Life Sciences International, Plymouth Meeting, PA), mouse anti-ER α (D-12), mouse anti- β -actin (C4), and goat anti-lamin B (C-20) (Santa Cruz Biotechnology, Santa Cruz, CA) were used as the primary antibodies. IP was performed using the Protein G HP SpinTrap (GE Healthcare, Buckinghamshire, UK) following the classic protocol. For each IP reaction, 4 μ g of anti-AhR antibody was immobilized to 100 μ l of protein G sepharose matrix before incubation with 200 μ g of nuclear extract. One-tenth of the eluate was analyzed by Western blotting against ER α .

Gene Expression Analysis

Transgenic cells were additionally treated with 1 nM E2, 10 nM TCDD, or both for 6 hours after an overnight treatment with or without 1 μ g/ml Dox. RNA extraction and real-time RT-PCR were performed as

Figure 2. Effects of 17 β -estradiol (E2) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on the transactivation activity of ER α (A and B) and AhR (C and D) in tetracycline-on (TO) cell lines: CL1-5(TO-ER α), CL1-5(TO-AhR), and CL1-5(TO-AhR/ER α). After estrogen-responsive element (ERE)-luciferase (Luc) or dioxin-responsive element (DRE)-Luc reporter transfection, cells were treated with various combinations of 10 nM TCDD and 1 nM E2 for 24 hours in the absence and presence of ER α and AhR transgene induction by 1 μ g/ml Dox ($n = 3$). * $p < 0.05$, ** $p < 0.005$.

described previously (29). Gene expression levels were normalized to β -actin expression levels.

ChIP Analysis

CL1-5(TO-AhR/ER α)#11 cells were treated with 1 nM E2, 10 nM TCDD, or both for 90 minutes after induction of transgene expression overnight with 1 μ g/ml Dox. DNA-protein cross-linking, DNA shearing, IP, and cross-link reversal were performed following the instruction manual of the Magna ChIP A kit (Millipore, Billerica, MA). Rabbit anti-AhR (H-211X) and anti-ER α (HC-20) (Santa Cruz Biotechnology) were used to precipitate the DNA fragments cross-linked to the respective antigens, whereas normal rabbit IgG (Santa Cruz Biotechnology) was used to detect background levels. Precipitated DNA was PCR quantified using the StepOne Plus Real-Time PCR system (Applied Biosystems, Carlsbad, CA) after reversal of cross-linking. DNA binding was calculated as percent of starting Ch following the Percent Input Method (Life Technologies) with background subtraction. PCR primer sequences are listed in the online supplement.

Statistical Analysis

All experiments were run in replicates. The number of replication is indicated in the figure legends. Results are presented as mean ($\pm$ SE). The significance of a difference was calculated using one-way ANOVA plus Scheffe's *post hoc* test (SPSS, Chicago, IL).

RESULTS

Construction of Inducible AhR/ER α -Expressing Cell Lines

In contrast to ER α , AhR is commonly present in lung epithelial cells, despite being at different levels (30). To assess dioxin and estrogen signaling in different AhR/ER α phenotypes of lung cells, we employed the AhR-deficient ER α -negative CL1-5 human lung adenocarcinoma cell line to construct cell lines that conditionally

express AhR, ER α , or both. Before transformation, AhR is not readily detectable in CL1-5 cells by Western blotting, except under conditions of overexposure. Upon stable transfection with the tetracycline-regulated TO-AhR expression system, AhR protein is highly expressed after a 24-hour treatment with 1 μ g/ml Dox (a tetracycline analog). A similar induction of ER α expression by Dox is seen with TO-ER α stable transfection (Figure 1). For each type of transformation, two daughter cell lines were selected based on Western blotting and reporter transfection.

Effects of E2 and TCDD on ER α and AhR Transactivation Activities

The transactivation activities of the ER α and AhR transgenes in the CL1-5 daughter cell lines were examined by transient transfection of ERE-Luc and DRE-Luc reporters, respectively. The enzyme activities of the reporters, reflecting reporter expression levels, were determined in the absence and presence of transgene induction (1 μ g/ml Dox for 24 h). One-way ANOVA plus Scheffe's *post hoc* analysis indicated that 1 nM E2 (a physiological concentration in women) exhibited a significant positive effect on ERE-Luc reporter activity in CL1-5(TO-ER α) and CL1-5(TO-AhR/ER α) cells only when ER α expression was induced by Dox (Figure 2). TCDD at 10 nM (an effective concentration frequently tested *in vitro*) had no effect on ERE-Luc reporter expression in CL1-5(TO-ER α) cells by itself, but reduced Dox/E2-stimulated ERE-Luc reporter activity by 17.7 ($\pm$ 5.1) to roughly 36.6 ($\pm$ 3.5)% (Figure 2A). When AhR expression was induced together with ER α , TCDD treatment lowered both basal and E2-stimulated ERE-Luc reporter activities. However, the decreases lacked statistical significance (Figure 2B).

Figure 3. Response of cytochrome P450 (CYP) 1B1 promoter to E2 and TCDD in CL1-5(TO-AhR) (A), CL1-5(TO-ER α) (B), and CL1-5(TO-AhR/ER α) (C). Indicated cell lines were transiently transfected with a CYP1B1-Luc reporter that harbored both dioxin- and estrogen-responsive elements in the promoter. The transfected cells were then treated for 24 hours with 10 nM TCDD, 1 nM E2, or both in the presence of 1 μ g/ml Dox ($n = 3$). * $P < 0.05$, ** $P < 0.005$.

Induction of AhR expression drastically activated basal DRE-Luc reporter activity in CL1-5(TO-AhR) and CL1-5(TO-AhR/ER α) cells. As compared with the Dox⁻/TCDD⁻ control,

Dox-induced TCDD-independent reporter activation was 7.54 (± 0.58) to roughly 9.08 (± 0.51)-fold in CL1-5(TO-AhR) and 34.70 (± 0.35) to roughly 55.80 (± 5.42)-fold in CL1-5(TO-AhR/ER α). Addition of 10 nM TCDD only roughly doubled Dox-induced DRE-Luc reporter activity (Figures 2C–2D). The results suggest that AhR was highly active in lung adenocarcinoma cells, regardless of the presence and absence of exogenous ligands. Addition of 1 nM E2 had no obvious effect on basal and TCDD-stimulated DRE-Luc reporter activity in Dox-treated CL1-5(TO-AhR) cells, but the response of the DRE-Luc reporter to TCDD became statistically insignificant in the presence of E2 (Figure 2C). When ER α was expressed alongside AhR, E2 diminished basal DRE-Luc reporter activity by near 50% and TCDD-stimulated reporter activity by 20.2 (± 1.0) to roughly 43.7 (± 2.0)% (Figure 2D).

Effects of E2 and TCDD on Neighboring ER α and AhR Binding Sites

CYP1B1 is an estrogen/dioxin dual-responsive gene that contains at least one ERE and two DRE enhancers within the 1,111-bp upstream region (31). We cloned the 1,111-bp CYP1B1 promoter in front of a Luc reporter gene and transfected the resulting CYP1B1-Luc reporter plasmid into all the CL1-5 daughter cell lines. In Dox-induced CL1-5(TO-AhR) cells, the 1,111-bp CYP1B1 promoter positively responded to TCDD treatment, but not to E2 treatment. Although E2 seemed to have no effect on TCDD-elicited CYP1B1-Luc activity in CL1-5(TO-AhR), the increases raised by TCDD lost statistical significance in the presence of E2 (Figure 3A). Treating Dox-induced CL1-5(TO-ER α) cells with 1 nM E2 significantly increased CYP1B1-Luc reporter activity. In contrast, 10 nM TCDD had no effect on CYP1B1-Luc reporter activity in CL1-5(TO-ER α) cells, regardless of E2 levels (Figure 3B). When expression of AhR and ER α was simultaneously induced in CL1-5(TO-AhR/ER α), 10 nM TCDD and 1 nM E2 each alone could increase CYP1B1-Luc reporter activity. However, the response to E2 was stronger than to TCDD. Cotreating Dox-induced CL1-5(TO-AhR/ER α) with TCDD and E2 raised reporter activity to a level similar to that from treatment with E2 alone (Figure 3C). The E2 $\cdot$ ER α action appeared to be predominant in the 1,111-bp CYP1B1 promoter when AhR and ER α were coexpressed.

Effects of E2, TCDD, and Cotreatment on mRNA Expression of Endogenous Responsive Genes

In addition to the effects on AhR and ER α transactivation activities at the responsive DNA sites, we examined regulation of endogenous responsive gene expression by E2 and TCDD (Figure 4). Responsive genes to be examined are proteinase inhibitor 9 (PI-9), CYP1A1, and CYP1B1. Whereas PI-9 is primarily regulated by estrogen in human liver cells (32), CYP1A1 and CYP1B1 are responsive to both estrogen and dioxin (16, 31). DRE sites are found in the proximal upstream regions of both CYP1A1 and CYP1B1, but the ERE motif is only located in the CYP1B1 promoter (31, 33).

In the absence of ER α expression, PI-9 mRNA level was low and steady after 6 hours of treatment with either 1 nM E2 or 10 nM TCDD in all three types of CL1-5 daughter cell lines. When ER α expression was induced by Dox in CL1-5(TO-ER α) and CL1-5(TO-AhR/ER α) cells, PI-9 mRNA expression was up-regulated. However, only the up-regulation in CL1-5(TO-AhR/ER α) exhibited statistical significance. Addition of E2 further increased PI-9 mRNA levels in ER α -expressing CL1-5(TO-ER α) and CL1-5(TO-AhR/ER α) cells. TCDD alone did not affect PI-9 expression in both CL1-5(TO-ER α) and CL1-5

Figure 4. Transcriptional expression of PI-9, CYP1A1, and CYP1B1 under different contexts of ER α and AhR. The mRNA levels of PI-9 (A–C), CYP1A1 (D–F), and CYP1B1 (G–I) in CL1-5(TO-AhR) (A, D, and G), CL1-5(TO-ER α) (B, E, and H), and CL1-5(TO-AhR/ER α) (C, F, and I) were measured by real-time RT-PCR ($n = 5$). Dox (1 μ g/ml) was added to cell lines 16 hours before a 6-hour treatment with 10 nM TCDD, 1 nM E2, or both. Expression levels of these three genes in each sample were normalized to β -actin expression level. * $P < 0.05$, ** $P < 0.005$.

(TO-AhR/ER α). Induction of AhR expression also failed to render PI-9 responsive to TCDD in either CL1-5(TO-AhR) or CL1-5(TO-AhR/ER α). However, TCDD/E2 cotreatment significantly decreased E2 $\cdot$ ER α -induced PI-9 expression in CL1-5(TO-ER α)#18 (Figures 4A–4C).

In contrast to PI-9, induction of AhR expression significantly raised CYP1A1 mRNA expression in CL1-5(TO-AhR) cells.

TCDD treatment further increased Dox-induced CYP1A1 expression. E2 had no effect on either basal or TCDD-stimulated CYP1A1 expression in Dox-induced CL1-5(TO-AhR) (Figure 4D). Although no functional ERE site had been located in the human CYP1A1 promoter to date, the CYP1A1 mRNA expression pattern indicated that CYP1A1 was positively regulated by E2 $\cdot$ ER α in human lung adenocarcinoma cells.

Figure 5. Nuclear localization, protein–protein interaction, and promoter occupancy of AhR and ERα. (A) CL1–5(TO-AhR/ERα)#11 was incubated overnight with 1 μg/ml Dox to induce expression of AhR and ERα recombinant proteins. Nuclear distribution of AhR and ERα in response to a 30-minute treatment with 1 nM E2, 10 nM TCDD, or both was examined by Western blot analysis of the nuclear extracts. Lamin B was used as a nuclear house-keeping marker. (B) The nuclear extracts described here were immunoprecipitated (IP) against AhR, followed by Western blotting (WB) against ERα. AhR had a protein–protein interaction with ERα in the presence of E2. (C) The ERα protein precipitated along with AhR in the IP–Western assays ($n = 3$) were quantified by the ERα:AhR ratio using densitometry. (D–I) Chromatin IP (ChIP) was employed to examine the residence of AhR and ERα in the indicated promoter regions in Dox-induced CL1–5(TO-AhR/ERα)#11 after 90 minutes of treatment with TCDD, E2, or both. A total of 1% of the chromatin used in the ChIP reaction was saved as the “input” control, whereas normal IgG was used to determine background binding. (D–F) Representative electrophoresis images of the ChIP assays. (G–I) Quantitative results determined by real-time PCR ($n = 4$). * $P < 0.05$, ** $P < 0.005$ compared with the basal condition; # $P < 0.05$ between groups indicated.

Addition of E2 greatly increased CYP1A1 mRNA levels in Dox-induced CL1–5(TO-ERα) cells. TCDD treatment had no effect on Dox/E2-stimulated CYP1A1 expression in CL1–5(TO-ERα) (Figure 4E). Induction of AhR together with ERα downgraded the TCDD responsiveness of CYP1A1 to an insignificant level. Even so, TCDD and E2 cumulatively increased CYP1A1 mRNA expression in Dox-induced CL1–5(TO-AhR/ERα) (Figure 4F).

The transcriptional changes of CYP1B1 in response to Dox, TCDD, and E2 treatments resembled those of CYP1A1, except that the scale of the response, particularly to E2, was smaller than that for CYP1A1 (Figures 4D–4I). It was likely that high basal expression of CYP1B1 in lung adenocarcinoma cells minimized the values of fold induction, hence lowering the relative sensitivity of this gene to TCDD and E2. Although CYP1B1 exhibited a significant response to TCDD treatment in Dox-induced CL1–5(TO-ERα) cells, endogenous AhR, rather than transgenic ERα, was responsible for the TCDD-induced CYP1B1 up-regulation because similar levels of up-regulation were detected in

Dox-treated and untreated cells (Figure 4H). In addition, we observed an interesting phenomenon that TCDD enhanced Dox/E2-induced CYP1B1 expression in CL1–5(TO-ERα)#17, but not in CL1–5(TO-ERα)#18 (Figure 4H), whereas TCDD antagonized Dox/E2-induced PI-9 expression in CL1–5(TO-ERα)#18, but not in CL1–5(TO-ERα)#17 (Figure 4B). It seems that the strength of TCDD in inhibition of E2-activated ERα activity determined the combined effect of E2 and TCDD on neighboring ERE and DRE sites in cells expressing abundant ERα, but little AhR.

Effects of E2, TCDD, and Cotreatment on the Nuclear Localization, Protein–Protein Interaction, and Promoter Occupancy of AhR and ERα

Nuclear localization is a prerequisite for the genomic actions of AhR and ERα. Western blot analysis of CL1–5(TO-AhR/ERα) #11 nuclear extracts showed that AhR and ERα could both enter the nuclei in a ligand-independent manner. A 30-minute

TABLE 1. EFFECT OF 2,3,7,8-TETRACHLORODIBENZO-*p*-DIOXIN ON ESTROGEN RECEPTOR α TRANSACTIVATION ACTIVITY

AhR/ER α		Basal Activity		E2-Induced Activity	
		ERE-Luc	CYP1B1-Luc	ERE-Luc	CYP1B1-Luc
$\pm$	+	—	—	$\downarrow$	—
+	+	—	ud	—	—

Definition of abbreviations: AhR, aryl hydrocarbon receptor; CYP, cytochrome P450; DRE, dioxin-responsive element; E2, 17 β -estradiol; ER, estrogen receptor; ERE, estrogen-responsive element; Luc, luciferase; ud, unable to determine.

Symbols represent the following: $\pm$, little expression; +, high expression; $\downarrow$, inhibition; —, no effect or response.

treatment with 10 nM TCDD and 1 nM E2 increased the nuclear distribution of AhR and ER α , respectively (Figure 5A). IP of the nuclear AhR protein, followed by Western blotting against ER α , revealed that, in presence of E2, ER α had a protein–protein interaction with AhR. Cotreatment with TCDD diminished the AhR–ER α interaction (Figures 5B and 5C).

We also examined promoter recruitment by ChIP. The residence of AhR in the –1,142/–791 DRE-containing region of CYP1B1 was detected in the absence of TCDD. TCDD treatment (10 nM for 90 min) increased the recruitment of AhR to the –1,142/–791 region. E2 (1 nM) alone or together with TCDD had no obvious effect on the AhR recruitment. ANOVA indicated different extents of AhR binding between treatments ($P = 0.027$). However, the differences had no statistical significance in the *post hoc* pairwise comparison. ER α was sometimes detected in the –1,142/–791 CYP1B1 promoter region under E2 and TCDD/E2 treatments. The presence of ER α was likely an artifact arising from Ch fragments containing both DRE and ERE (Figures 5D and 5G). ER α and AhR were found in the –211/–18 ERE-containing region of CYP1B1 in the presence of E2, although the binding level of AhR was far lower than that of ER α (Figures 5E and 5H). If not an artifact, AhR was probably recruited to this ERE site through a direct interaction with E2 · ER α , as suggested by IP–Western blot data (Figure 5B). Cotreatment with TCDD did not significantly decrease the binding of ER α to the –211/–18 site. However, the binding level lost statistical significance as compared with the basal control (Figures 5E and 5H). AhR exhibited similar levels of binding to the –632/–414 CYP1A1 DRE enhancer under all treatments. To our surprise, we found that ER α was recruited to the –632/–414 CYP1A1 region in the presence of E2. Comparable levels of ER α binding were detected under E2 and TCDD/E2 treatments (Figures 5F and 5I).

DISCUSSION

This study investigated the interplay of TCDD and E2 in lung adenocarcinoma cells with different AhR/ER α expression patterns. Tables 1–3 summarize the mutual effects of TCDD and E2 on ER α - and AhR-mediated signaling based on reporter activities and endogenous gene expression. Reporter transfection data suggest that TCDD acted as a weak antagonist against E2-activated ER α activity in lung adenocarcinoma cells that expressed abundant ER α , but little AhR (i.e., AhR $^+$ /ER α $^+$; Table 1). Because TCDD cannot displace E2 from ER α (34), it is unlikely that TCDD antagonized ER α by competition with E2 as a ligand. Another mechanism was involved. The mRNA expression profile of PI-9 revealed that the antiestrogenic activity of TCDD only occurred in CL1–5(TO-ER α)#18, but not in CL1–5(TO-ER α)#17 (Table 3). The discrepancy probably arose from differences in promoter structure. Whereas the ERE-Luc reporter containing three consecutive ERE elements in front of a basal promoter bluntly amplified the transactivation activity of

ER α under treatments, the endogenous PI-9 gene was regulated by a more intricate mechanism. TCDD antiestrogenicity might vary with the cellular AhR:ER α ratio, because: (1) TCDD did not inhibit E2 from activating ERE-Luc and PI-9 expression in cells expressing high levels of AhR and ER α (AhR $^+$ /ER α $^+$); and (2) CL1–5(TO-ER α)#18 exhibited higher ER α expression and E2 responsiveness than CL1–5(TO-ER α)#17. Stronger TCDD antiestrogenicity was also observed in E2-sensitive ECC-1 endometrial cancer cells compared with less sensitive Ishikawa endometrial cancer cells (35, 36). TCDD might have lost antiestrogenicity when the expression level of AhR relative to ER α rose above a certain level.

In contrast, E2 in association with ER α had a negative effect on basal and TCDD-induced AhR activity in lung adenocarcinoma cells (Table 2). Because ER α and AhR had a protein–protein interaction and coexisted at the ERE enhancer in the presence of E2, E2 · ER α might inhibit AhR activity by hijacking AhR away from DRE. When ERE and DRE were located in proximity (e.g., CYP1B1), mutual inhibition by TCDD and E2 disappeared in AhR $^+$ /ER α $^+$ cells (Tables 1 and 2). Furthermore, TCDD and E2 cumulatively increased CYP1B1 mRNA expression in AhR $^+$ /ER α $^+$ cells (Table 3). Taking ChIP results into account, TCDD and E2 seemed to separately increase the binding of AhR and ER α to the corresponding enhancers and raise CYP1B1 mRNA expression in parallel. In AhR $^+$ /ER α $^+$ CL1–5(TO-ER α)#18 cells, the negative effect of E2 on AhR activity might still exist at the CYP1B1 DRE enhancer, rendering the gene unresponsive to TCDD during cotreatment. Increase of the relative expression of AhR to ER α (e.g., CL1–5(TO-ER α)#17) seemed to remove potential inhibition by E2 · ER α (Table 3).

Compared with CYP1B1, CYP1A1 was more sensitive to E2 in ER α $^+$ lung adenocarcinoma cells, despite lacking a characteristic upstream ERE sequence. E2 enhanced the responsiveness of CYP1A1 to TCDD in AhR $^+$ /ER α $^+$ lung adenocarcinoma cells (Table 3). However, controversy exists over the role of ER α in regulation of CYP1A1. Matthews and colleagues (16) showed that ER α was required for a full-scale CYP1A1 induction by TCDD in T47D human breast cancer cells. Cotreatment with TCDD and E2 increased the recruitment of ER α to the CYP1A1 promoter, but had no effect on TCDD-induced CYP1A1 mRNA expression. Wihlén and colleagues (37) showed that ER α was dispensable for the TCDD responsiveness of CYP1A1 in MCF-7 human breast cancer cells, whereas Beischlag and Perdew (14) showed that ER α was recruited to the CYP1A1 promoter as a transcriptional corepressor in TCDD/E2-cotreated MCF-7 cells. On the other hand, Klinge and colleagues (15) showed that E2 stimulated basal reporter activation from the CYP1A1 promoter, but had no effect on TCDD-induced activation in MCF-7 cells. Even so, regulation of CYP1A1 by estrogen may exist in the lung, because significantly higher levels of CYP1A1 mRNA were detected in the

TABLE 2. EFFECT OF 17 β -ESTRADIOL ON ARYL HYDROCARBON RECEPTOR TRANSACTIVATION ACTIVITY

AhR/ER α		Basal Activity		TCDD-Induced Activity	
		DRE-Luc	CYP1B1-Luc	DRE-Luc	CYP1B1-Luc
+	—	—	—	—	—
+	+	$\downarrow$	ud	$\downarrow$	—

Definition of abbreviations: AhR, aryl hydrocarbon receptor; CYP, cytochrome P450; DRE, dioxin-responsive element; ER, estrogen receptor; Luc, luciferase; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; ud, unable to determine.

Symbols represent the following: —, no expression; +, high expression; $\downarrow$, inhibition; —, no effect or response.

TABLE 3. GENE EXPRESSION IN RESPONSE TO 17 β -ESTRADIOL AND 2,3,7,8,-TETRACHLORODIBENZO-*p*-DIOXIN TREATMENTS

AhR/ER α	Response to E2			Effects of TCDD on E2 Responsiveness			Response to TCDD			Effects of E2 on TCDD Responsiveness		
	PI-9	CYP1A1	CYP1B1	PI-9	CYP1A1	CYP1B1	PI-9	CYP1A1	CYP1B1	PI-9	CYP1A1	CYP1B1
+	—	—	—	—	—	—	—	↑	↑	—	—	—
±	+	↑	↑	—/↓	—	C/—	—	—	↑	—	—	C/M
+	+	↑	↑	—	C	C	—	—	↑	—	↑	C

Definition of abbreviations: AhR, aryl hydrocarbon receptor; C, cumulative response; CYP, cytochrome P450; E2, 17 β -estradiol; ER, estrogen receptor; M, masking responsiveness; PI-9, proteinase inhibitor 9; TCDD, 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin.

Symbols represent the following: —, no expression; ±, little expression; +, high expression; ↑, stimulation; ↓, inhibition; —, no effect or response.

nontumor lung tissues from female patients than in that from male patients (8).

In MCF-7 and T47D breast cancer cells, the binding of ER α to the –1,125/–980 CYP1A1 dioxin-responsive enhancer requires the presence of TCDD · AhR (16). Our ChIP analysis showed that ER α resided in the –632/–414 dioxin-responsive region of CYP1A1 in lung adenocarcinoma cells only when associated with E2. Cotreatment with TCDD did not affect the occupancy of E2 · ER α . If E2 · ER α were recruited to the –632/–414 CYP1A1 promoter region by association with AhR, TCDD/E2 cotreatment should have decreased the occupancy of E2 · ER α in this promoter region due to a reduced protein–protein interaction between AhR and ER α . In addition, the binding level of ER α was much higher than AhR at this site. Our results suggest an AhR-independent recruitment of E2 · ER α to the –632/–414 CYP1A1 promoter region. E2 has been found to induce *c-fos* and *cad* gene expression through ER α –Sp1 interaction at the GC-rich Sp1 site (38, 39). GC-rich motifs have been located near the DRE enhancers in the CYP1A1 promoter (40). It is possible that E2 · ER α regulates CYP1A1 through a similar ER α –Sp1 interaction in lung adenocarcinoma cells; however, this assumption needs to be proved.

CYP1A1 and CYP1B1 are two major CYP enzymes responsible for phase I estrogen metabolism in extrahepatic tissues (41).

Induction of CYP1A1 and CYP1B1 in response to E2 imposes a feedback regulation on estrogen signaling in ER α -expressing lung cells. Meanwhile, CYP induction increases the chance of carcinogenesis if hydroxylated estradiols or other reactive CYP metabolites are not rapidly metabolized by phase II enzymes. It is generally believed that metabolic activation into reactive metabolites by CYPs is important for tumor initiation by carcinogens, such as benzo[a]pyrene (42). Cumulative up-regulation of CYP1A1 and CYP1B1 by TCDD and E2 in AhR⁺/ER α ⁺ cells may cause a greater risk of carcinogenesis by accelerating unbalance between carcinogen activation and detoxification. This may explain, in part, the positive trends with increased serum estrogen and tumor ER α levels for non-small-cell lung cancer (9, 13).

Halogenated dioxins and furans are not readily metabolized as PAHs. The parental compounds and metabolites of dioxins and furans also cannot react with DNA to cause damage (1). Nevertheless, it has been shown that repeated treatment with low doses of TCDD, not high, toxic doses, increases the multiplicity of lung tumors initiated by genotoxin (43). A constitutively active AhR transgenic mouse model confirms that persistent AhR activation promotes tumor formation after initiation by genotoxin (44). Our previous study also showed that CL1–5(TO-AhR) cells had increased anchorage-dependent and -independent proliferation after pretreatment with a condensate of cigarette sidestream smoke

Figure 6. Proposed genomic actions of dioxin and estrogen in lung cells. AhR is commonly present in lung cells, but at different expression levels. Dioxin can displace endogenous ligands to enhance the transactivation activity of AhR. Estrogen-bound ER α diminishes basal and dioxin-induced AhR activity by titrating AhR away from DRE via a protein–protein interaction. Dioxin may antagonize ER α activity when AhR expression is minimal. Increase of AhR expression counteracts the reciprocal inhibitory effects of dioxin and estrogen. Dioxin and estrogen cumulatively increase expression of dual-responsive genes in AhR⁺/ER α ⁺ lung cells.

particulates (28). In addition to metabolic genes, AhR, as well as ER α , regulates a spectrum of genes involved in proliferation, differentiation, motility, and migration (4, 45). AhR and ER α have carcinogenic potential upon constantly activated.

Compatible with the carcinogenic potential of AhR, neoplastic lung cells, especially adenocarcinoma cells, are inclined to have higher levels of AhR expression than normal lung cells (30). In this study, we also found that AhR was highly active in human lung adenocarcinoma cells. AhR could translocate into the nucleus and bind to its responsive site in the absence of exogenous ligands. Treating CL1-5(TO-AhR) cells with the AhR antagonist, 3',4'-dimethoxyflavone, diminished basal and inducible DRE-Luc activation (see Figure E1 in the online supplement). High basal AhR activity is not limited to CL1-5(TO-AhR) cells. Knockdown of AhR expression in A549 and H838 lung adenocarcinoma cells by RNA interference also removed more than 90% of the intrinsic activity (Figure E2). Endogenous ligands may contribute to the high basal AhR activity. The higher AhR activation potency and lower CYP1A1 expression exhibited by AhR-null mouse lung tissues compared with wild-type counterparts support the presence of CYP-metabolizable endogenous AhR ligands in the lung (46). A number of endogenous metabolites have been proposed to be potential AhR ligands (47); among them, 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester has been identified in porcine lung tissues (48). Although high basal activity lowers the magnitude of activity induction stimulated by TCDD, sustained AhR activation by either endogenous or exogenous ligands may render cells more susceptible to carcinogens.

The present study confirms that the genomic action of dioxins and dioxin-like compounds in lung cells varies with estrogen level, AhR/ER α expression pattern, and promoter context. Based on our results, we propose that AhR is active in lung cells, probably due to the presence of endogenous ligands. Dioxin exposure further enhances AhR activation. When AhR expression is minimal, dioxin may antagonize estrogen-activated ER α transactivation activity. In contrast, estrogen has no effect on either ER α or AhR target genes in the absence of ER α . When bound to ER α , estrogen diminishes basal and dioxin-induced AhR transactivation activity, perhaps by titrating AhR away from its binding site via AhR-ER α protein interaction. Coexistence of ERE and DRE in a promoter or increase of the relative expression of AhR to ER α counteracts the reciprocal inhibitory effects of dioxin and estrogen on the ER α and AhR signaling pathways. Moreover, dioxin and estrogen cumulatively up-regulate expression of dual responsive genes, such as CYP1A1 and CYP1B1, in lung cells expressing high levels of AhR and ER α (Figure 6). Aside from the classical pathways, we should keep in mind that AhR and ER α can exert their effects via a variety of nonclassical mechanisms. Dioxin-like compounds that also modulate ER α as a ligand add additional complexity to the AhR-ER α interaction. Although dioxins and furans are usually released to the environment at very low concentrations, their widespread distribution and long half-lives still evoke concern about cumulative risk of lung cancer. Chronic exposure to low concentrations of dioxins and dioxin-like furans and PAHs may increase lung cancer risk, particularly among people who have high AhR/ER α expression and elevated estrogen milieu in the lung by promotion of metabolic activation of carcinogens and outgrowth of neoplastic cells.

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