Co-planar 3,3',4,4',5-pentachlorinated biphenyl and non-co-planar 2,2',4,6,6'-pentachlorinated biphenyl differentially induce recruitment of oestrogen receptor α to aryl hydrocarbon receptor target genes

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In the present study we examined the ability of 3,3',4,4',5-pentachlorinated biphenyl [PCB126 (polychlorinated biphenyl 126)], a prototypical AHR (aryl hydrocarbon receptor) agonist, and 2,2',4,6,6'-PCB (PCB104), which does not activate AHR, to induce the recruitment of ER α (oestrogen receptor α) to *CYP1A1* (cytochrome P4501A1 gene) and *CYP1B1* promoters in T-47D human breast cancer cells and other cell lines. PCB126 treatment strongly induced *CYP1A1* and *CYP1B1* mRNA expression that was unaffected by co-treatment with E2 (17 β -oestradiol). PCB104 failed to induce changes in either *CYP1A1* or *CYP1B1* expression levels. ChIP (chromatin immunoprecipitation) assays show that PCB126, but not PCB104, increased the promoter occupancy by ER α to *CYP1A1* and *CYP1B1* promoters. Co-treatment with PCB126 + E2 significantly enhanced the promoter occupancy of ER α at *CYP1A1*, whereas co-treatment with PCB126 + 4-hydroxytamoxifen or ICI182,780 did not. Competitive binding studies revealed that neither PCB126 nor PCB104 bound to ER α . HEK-293 cells (human embryonic kidney-293

cells) stably transfected with ER α showed significantly higher PCB126-induced *CYP1A1* expression compared with empty vector controls, whereas no increase was observed in cells stably transfected with ER α lacking its N-terminal AF1 (activation function-1) domain (ER α Δ AF1). Despite no increase in AHR-mediated gene expression, ChIP assays revealed that ER α Δ AF1 was present at *CYP1A1* and *CYP1B1* promoters. HC11 mouse mammary cells stably expressing shRNA (small-hairpin RNA) against ER α showed an 8-fold reduction in PCB126-dependent *Cyp1a1* expression. Our results provide further evidence that AHR agonists induce ER α promoter occupancy at AHR target genes through indirect activation of ER α , and support a role for ER α in AHR transactivation.

Key words: aryl hydrocarbon receptor (AHR) target gene, chromatin immunoprecipitation (ChIP), cytochrome P450 (CYP), oestrogen receptor α (ER α), polychlorinated biphenyl (PCB), small-hairpin RNA.

INTRODUCTION

The AHR (aryl hydrocarbon receptor) binds and mediates most, if not all, of the toxic effects of TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) [1,2]. A number of other structurally related halogenated compounds including PAHs (polycyclic aromatic hydrocarbons) and co-planar PCB (polychlorinated biphenyls) also bind and activate the AHR [1]. After ligand binding, the cytoplasmic AHR translocates to the nucleus where it binds its obligatory dimerization partner ARNT (AHR nuclear translocator). The activated AHR–ARNT heterodimer complex binds to its cognate DNA sequences, termed XREs (xenobiotic-response elements), located in the upstream regulatory region of target gene promoters. Binding of the AHR–ARNT heterodimer induces recruitment of co-regulator proteins and chromatin remodelling factors, resulting in changes in the expression of AHR target genes, such as *CYP1A1* (cytochrome P4501A1) and *CYP1B1* [3,4].

The endocrine disruptive effects of TCDD and related compounds are well documented [5–8], but perhaps most studied in regard to their antioestrogenic activity [7,9]. The molecular

basis for AHR/ER α (oestrogen receptor α)-inhibitory cross-talk is unclear and has been proposed to be due to a combination of several different mechanisms including increased synthesis of inhibitory factors [10], direct inhibition through inhibitory XREs located in oestrogen-responsive gene promoters [11], sequestration of common cofactors [12,13], increased ER degradation [14] and altered E2 (17 β -oestradiol) metabolism [15].

Oestrogen action is mediated by two ligand-activated transcription factors, ER α and ER β , which are members of the NR (nuclear receptor) superfamily of transcription factors [16]. Although ligand binding is an important mechanism of NR activation, ERs are also activated by growth hormones, kinases and other hormone-bound steroid receptors and transcription factors [17,18]. ERs regulate gene expression in two ways: via direct DNA binding to EREs (oestrogen-response elements) or by protein–protein interactions with other transcription factors [19,20]. Recent data from our group revealed that, in the absence of E2, TCDD-bound AHR recruits ER α to the AHR-regulated genes *CYP1A1* and *CYP1B1* [8]. This recruitment most likely occurs through direct protein–protein interactions between ER α

Abbreviations used: 4OH-TAM, 4-hydroxytamoxifen; AF1, activation function-1; AHR, aryl hydrocarbon receptor; ARNT, AHR nuclear translocator; ChIP, chromatin immunoprecipitation; CYP, cytochrome P450; *CYP1A1*, cytochrome P4501A1 gene; DCC, dextran-coated charcoal; DMEM, Dulbecco's modified Eagle's medium; E2, 17 β -oestradiol; EGF, epithelial growth factor; ER, oestrogen receptor; ERE, oestrogen-response element; FBS, fetal bovine serum; FRT, Flp (flippase) recombination target; HEK-293 cells, human embryonic kidney cells; hER α , human ER α ; HRP, horseradish peroxidase; NF- κ B, nuclear factor κ B; LBD, ligand-binding domain; NR, nuclear receptor; PCB, polychlorinated biphenyl; qRT-PCR, quantitative real-time PCR; shRNA, small-hairpin RNA; SERM, selective ER modulator; shER α , shRNA targeting ER α ; STAT5, signal transducer and activator of transcription 5; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TNF α , tumour necrosis factor α ; XRE, xenobiotic-response element.

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and activated AHR [21,22]. A number of 'traditional' AHR ligands have recently been shown to directly activate ER α , giving rise to a class of bifunctional AHR/ER α agonists [21,23,24] and to the possibility that direct activation of ER α is required to induce binding of ER α to AHR target gene promoters. However, in our previous studies, E2 did not induce the recruitment of ER α to AHR-regulated promoters, suggesting that an activated AHR is required to mediate this process [8]. Therefore whether recruitment of ER α to AHR target gene promoters is via direct ER α activation by AHR ligands or via indirect activation by activated AHR is unclear. Moreover, the physiological relevance of the oestrogenic activity of AHR agonists is unclear, since in the presence of E2, these ligands exhibit antioestrogenic activity [7].

PCBs are a family of 209 chemically related compounds that were widely used in a number of industrial applications. PCBs are persistent environmental pollutants that elicit a number of adverse health effects including teratogenesis, neurotoxicity, immunotoxicity, reproductive toxicity, endocrine disruption and carcinogenesis [25]. Two major structural classes of PCBs include the co-planar PCBs, which include several 'TCDD-like' PCBs such as 3,3',4,4',5-pentachlorinated biphenyl (PCB126) [26] and non-co-planar derivatives, which have been suggested to elicit oestrogenic activity [27,28]. PCB126 is one of the most toxic PCB congeners but is present at comparatively low concentrations in the environment [29]. However, relatively high levels of PCB126 have been reported in human milk samples [30].

In the present study, we investigated the ability of 3,3',4,4',5-pentachlorinated biphenyl (PCB126), a prototypical AHR agonist [26], and the non-co-planar 2,2',4,6,6'-PCB (PCB104), which exhibits oestrogen-like and not TCDD-like activity [27], to induce the recruitment of ER α to *CYP1A1* and *CYP1B1* promoters. We also examined the influence of ERs on AHR-dependent transcription. The results show that treatment with PCB126, but not PCB104, resulted in recruitment of ER α to AHR target genes. Promoter occupancy by ER α was enhanced by co-treatment with E2; however, no increases in *CYP1A1* or *CYP1B1* mRNA levels were observed following E2 co-treatment. Overexpression of ER α , but not an ER α variant lacking its N-terminal AF1 (activation function-1) (ER α Δ AF1) domain, enhanced PCB126-induced *CYP1A1* mRNA expression. ChIP (chromatin immunoprecipitation) assays revealed that both full-length ER α and ER α Δ AF1 were recruited to *CYP1A1* and *CYP1B1* promoters, suggesting that recruitment of ER α to AHR target genes may be independent of direct interaction of ER α with AHR. Stable knockdown of ER α significantly reduced PCB126-stimulated *CYP1A1* expression. These results indicate that AHR agonists induce *CYP1A1* promoter occupancy by ER α through indirect activation of ER α , and also support the notion of an important role for ER α in AHR transactivation.

MATERIALS AND METHODS

Chemicals and plasmids

Antibodies used in ChIP assays include: for ER α , H-184; for AHR, H-211; for ARNT, H-172 (all from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.). The anti-FLAG antibody was purchased from Sigma (St. Louis, MO, U.S.A.). TCDD, PCB126 and PCB104 were provided by Dr S. Safe (Institute of Biosciences and Technology, Texas A&M University, College Station, TX, U.S.A.), and E2 was from Sigma. All ligands were dissolved in DMSO. Cell culture media, media supplements and FBS (fetal bovine serum) were purchased from Invitrogen (Carlsbad, CA, U.S.A.). All other chemicals and biochemicals were of the highest quality available from commercial sources.

Generation of stable cell lines

Stable cell lines expressing hER α (human ER α) and hER α Δ AF1 respectively were generated using the Flp-In system (Invitrogen). HEK-293 (human embryonic kidney-293 cells) Flp-In cells were stably transfected with pcDNA5/FRT [where FRT is the Flp (flippase) recombination target; Invitrogen] containing cDNAs encoding hER α amino acids 1–595, hER α amino acids 180–595 or the empty vector (pcDNA5/FRT), generating the cell lines HEK-293-ER α , HEK-293-ER α Δ AF1 and HEK-293-FRT respectively. All cDNAs were N-terminally tagged with the FLAG epitope (MDYKDDDDK). Positive clones were selected with hygromycin (0.15 mg/ml). The generation and characterization of HC11 mouse mammary epithelial cells stably transfected with empty vector and shRNA (small-hairpin RNA) ER α respectively have been described previously [31].

Cell culture

T-47D human breast carcinoma cells were cultured in 1:1 mixture of DMEM (Dulbecco's modified Eagle's medium) and F12 Ham's nutrient mixture medium supplemented with 10% (v/v) FBS. HEK-293-ER α , HEK-293-ER α Δ AF1 and HEK-293-FRT were maintained in high-glucose DMEM supplemented with 10% FBS and 0.15 mg/ml hygromycin B. HC11 cells were maintained in RPMI 1640 containing 10% FBS, 5 μ g/ml insulin, 10 ng/ml EGF (epithelial growth factor) and 10 μ g/ml blasticidin S. All media were supplemented with 2 mM L-glutamine and 1% penicillin/streptomycin, and cells were maintained at 37°C and 5% CO₂.

ChIP

T-47D cells were seeded in 150-mm-diameter dishes or six-well plates and grown for 3 days in Phenol Red-free DMEM/F12 supplemented with 5% DCC (dextran-coated charcoal)-treated FBS (DCC-FBS). HEK-293 cells were seeded in six-well dishes and grown for 2 days in DMEM with 10% FBS. ChIP assays were performed as previously described [8]. ChIP DNA (5 μ l) was amplified by PCR with primers 5'-ACCCGCCACCTTCG-ACAGTTC-3' and 5'-TGCCCAGGCGTTGCGTGAGAAG-3' for *CYP1A1* enhancer region, 5'-CATGTCGGCCACGGAGTTTCT-TC-3' and 5'-ACAGTGCCAGGTGCGGTTCTTTC3' for the non-specific primers in the *CYP1A1* coding region, and 5'-GTGC-GCACGGAGGTGGCGATA3' and 5'-GCTCCTCCCGCGCTT-CTCAC3' for *CYP1B1*. For real-time PCR, SYBR Green qPCR supermix UDG (Invitrogen) was used to amplify a smaller fragment of the *CYP1A1* promoter as described previously [32], 5'-ATATGACTGGAGCCGACTTTCC-3' and 5'-GGCGAACT-TTATCGGGTTGA-3' for *CYP1B1*, and 5'-GGCCATCTCTCA-CTATGAATCACT-3' and 5'-GGATTGTGCTGATAGACAGAGA-CGA-3' for pS2. Results were normalized to zero time for each antibody and reported as fold enrichment of above zero time at the promoter. For the stable HEK-293 cells, promoter occupancy was determined relative to the input samples for each treatment.

Competitive ligand binding assay

Ligand binding was determined using an SPA (scintillation proximity assay) with streptavidin-coated polyvinyltoluene scintillation beads (GE Healthcare) and biotinylated receptor. The recombinant biotin-labelled LBDs (ligand-binding domains) of hER α and hER β were produced at high levels in *Escherichia coli* and extracted with a buffer containing 100 mM Tris/HCl (pH 8.0), 100 mM KCl, 10% (v/v) glycerol, 5 mM EDTA, 4 mM dithiothreitol and 0.1 mM PMSF (ER α -LBD extraction) with a microfluidizer. In all ligand binding experiments, the hER α -LBD

and hER β -LBD extracts were diluted in phosphate buffer {18 mM K₂HPO₄/2 mM KH₂PO₄, 20 mM Na₂MoO₄, 1 mM EDTA and 1mM TCEP [tris-(2-carboxyethyl)phosphine], pH 7.6} to a final concentration of 0.7 nM receptor. The [³H]E2 (PerkinElmer, Boston, MA, U.S.A.) had a specific radioactivity of 95 Ci/mmol. The final concentration of [³H]E2 used for competitive binding was 1.2 nM. Ligands were diluted from a 1 mM stock solution in DMSO in 12 concentrations ranging from 157 μ M to 38 pM by using a Hamilton Micro Lab AT2 PLUS robot. TCDD was diluted from a 0.1 mM stock solution. The incubation time on the shaker for the binding experiments was 20 h at room temperature (21–22°C). Receptor-bound [³H]E2 was determined by scintillation counting (PerkinElmer Trilux Microbeta). The IC₅₀ values were calculated using a four-parameter logistic equation:

$$y = \text{bottom} + (\text{top} - \text{bottom})/[1 + 10(x - \log \text{IC}_{50})]$$

in XLfit version 2.0 (ID Business Solutions).

RNA isolation and real-time PCR

T-47D cells were seeded in six-well plates and grown in a 1:1 mixture of Phenol Red-free DMEM and F12 Ham's nutrient mixture supplemented with 5 % DCC-FBS 24 h prior to ligand treatment. E2 (10 nM), PCB126 (1 μ M), PCB104 (1 μ M), or PCBs plus 10 nM E2 were added to cells for another 24 h or for the specified time. HC11 cells were seeded in six-well plates and grown in Phenol Red-free RPMI 1640 supplemented with 10 % DCC-FBS, 5 μ g/ml insulin, 10 ng/ml EGF and 10 μ g/ml blasticidin S 24 h prior to ligand treatment and treated for 24 h with ligands. HEK-293-ER α , HEK-293-ER α ΔAF1 and HEK-293-FRT cells were seeded in six-well plates and grown for 24 h in Phenol Red-free high-glucose DMEM supplemented with 10 % DCC-FBS and hygromycin B (0.3 mg/ml). Cells were then treated with ligands for 24 h. RNA was isolated using RNeasy spin columns (Qiagen) and reverse-transcribed as described previously [8]. One microgram of RNA was reverse-transcribed using Superscript II, and qRT-PCR (quantitative real-time PCR) was performed using 1 μ l of the cDNA synthesis reactions using SYBR Green (Invitrogen). All target gene transcripts were normalized to the 18 S rRNA (PE Applied Biosystems) content and to the DMSO or zero time samples.

Western blot

Proteins were resolved by SDS/10 % PAGE and transferred to a Hybond C-super membrane (Amersham) in 25 mM Tris base (pH 8.3) containing 19.2 mM glycine and 20 % (v/v) methanol. The membrane was blocked in 5 % fat-free milk overnight at 4°C with constant rocking and then incubated with a 1:2000 dilution of anti-ER α (Santa Cruz Biotechnology HC-20) antibody in 1 % fat-free milk PBS with 0.1 % Tween 20 for 1.5 h at room temperature. The membrane was then washed in PBS/Tween for 30 min and incubated with HRP (horseradish peroxidase)-conjugated anti-rabbit secondary antibody for 1 h at room temperature with constant rocking. After washing, the bands were visualized using SuperSignal West Pico chemiluminescent substrate (Pierce Chemical) according to the manufacturer's instructions. The membranes were exposed to an autoradiography film for 30 s to 2 min. For detection of β -actin, a 1:50000 dilution of primary mouse anti- β -actin antibody (Sigma) was incubated for 45 min at room temperature followed by a 1 h washing with PBS/Tween prior to incubation with HRP-conjugated anti-mouse secondary antibody for 1 h at room temperature. A final wash of 1 h in PBS/Tween was performed before development with SuperSignal West Pico chemiluminescent substrate.

Statistical analysis of data

Results of qRT-PCR experiments are presented as means $\pm$ S.E.M. for two or three independent experiments. Statistical comparisons were made using the two-tailed Student's *t* test where appropriate, or data were analysed by GraphPad Prism (San Diego, CA, U.S.A.); statistical analysis software was used for ANOVA. Comparisons of means by Student's *t* test or following a significant (*P* < 0.05) ANOVA test were determined by Tukey's multiple comparison test.

RESULTS

PCB126 but not PCB104 induces CYP1A1 and CYP1B1 expression

To demonstrate the functional differences between co-planar PCB126 and non-co-planar PCB104 in stimulating AHR-dependent gene expression and determine how E2 influences this activity, T-47D cells were treated with PCBs in the presence or absence of E2 and the mRNA expression levels of CYP1A1 and CYP1B1 were determined by qRT-PCR. As shown in Figure 1(A), induction of CYP1A1 mRNA was first detected after 60 min of treatment with PCB126, increased throughout the time course and was strongly induced after 24 h. Co-treatment with E2 did not significantly affect PCB126-induced CYP1A1 mRNA expression levels. CYP1A1 mRNA levels were not induced by PCB104 or PCB104 + E2. Similar results were obtained for CYP1B1 mRNA expression with a few notable differences (Figure 1A). Although the level of PCB126-induced CYP1B1 expression was two to three orders of magnitude lower than for CYP1A1, significant increases in CYP1B1 expression were detected after 30 min of treatment. Neither PCB104 treatment nor PCB104 + E2 co-treatment significantly increased CYP1B1 mRNA expression levels. ChIP assays showed that PCB126, but not PCB104, induced recruitment of AHR to *CYP1A1* and *CYP1B1* promoters (Figure 1B). No recruitment was observed to a region of the CYP1A1 mRNA. These results demonstrate the differential ability of PCB126 and PCB104 to induce AHR-dependent transcription.

ER α recruitment to AHR-regulated promoters is induced by PCB126 but not by PCB104

Our previous studies have shown that ER α is recruited by TCDD to *CYP1A1* and *CYP1B1* promoters, and that ER α recruitment is enhanced by co-treatment with E2 [8]. This suggests that AHR ligands, through their interactions with AHR, modulate ER α activity in a manner distinct from E2, causing its recruitment to AHR target genes. To test this hypothesis, ChIP assays were performed on cell extracts from T-47D cells exposed to PCBs in the presence or absence of E2 and the promoter enrichment of AHR, ARNT and ER α at *CYP1A1* and *CYP1B1* enhancer regions was determined. For these experiments, ChIP assays were analysed by highly sensitive qRT-PCR, since conventional PCR followed by gel electrophoresis may not accurately depict small changes among samples [8,33].

PCB126 treatment caused significant promoter enrichment of AHR and ARNT to *CYP1A1*, which reached a peak at 60 min and was maintained at a half-maximal level through 300 min (Figure 2). While not inducing any significant increases in CYP1A1 mRNA expression, PCB104 and PCB104 + E2 treatments resulted in small, but significant recruitment of AHR and ARNT to *CYP1A1*. Similar to that observed for CYP1A1, promoter enrichment of AHR and ARNT at *CYP1B1* reached a peak at 30 and 60 min. The level of fold enrichment at the *CYP1B1* promoter was similar to that at *CYP1A1* despite the relatively low

Figure 1 PCB126 and PCB104 differentially induce CYP1A1 and CYP1B1 mRNA expression

(A) Total RNA was isolated from T-47D human breast cancer cells treated with solvent (DMSO), 1 μ M PCB or 1 μ M PCB + 10 nM E2, DNase I-treated, and amplified with primers recognizing CYP1A1 or CYP1B1 mRNA. Results shown are means $\pm$ S.D. for at least two independent experiments. RNA expression levels significantly ($P < 0.05$) different between the PCB-treated and PCB + E2-treated time-matched samples are indicated by an asterisk. (B) Recruitment of AHR to CYP1A1 or CYP1B1 promoters was determined by ChIP assays, and analysis of the CYP1A1 open reading frame served as negative control.

ligand-dependent increase in CYP1B1 mRNA expression. No significant differences in the recruitment profiles of AHR and ARNT to CYP1A1 or CYP1B1 were observed by co-treatment with E2, with the exception of PCB104 + E2 at 120 min. Small but significant increases in the promoter occupancy by AHR and ARNT at CYP1B1 were observed in cells treated with PCB104 and PCB104 + E2 for 60 and 120 min. In agreement with our previous studies, PCB126 induced recruitment of ER α to CYP1A1 reaching a peak at 60 min and still present at the promoter after 300 min, albeit at lower levels [8]. The recruitment of ER α was enhanced by co-treatment with PCB126 + E2. In addition, PCB126 induced promoter enrichment of ER α to CYP1B1 that was also strongly increased by co-treatment with E2. The enhancement of ER α recruitment by PCB126 + E2 was transient and not observed, or was less pronounced, at 120 and 300 min.

Treatment with PCB104 alone did not induce recruitment of ER α to CYP1A1 or to CYP1B1. However, a small but significant increase in the promoter occupancy of ER α to CYP1B1 was observed at 30 min following PCB104 + E2 (Figure 2). In agreement with previous reports we observed that E2 induced recruitment of ER α to CYP1B1 [34], albeit at much lower levels than that induced by PCB126 or PCB126 + E2 treatment. This would suggest that the recruitment of ER α to CYP1B1 was not due to PCB104 but rather, and most likely, a result of an imperfect ERE palindrome located in the CYP1B1 promoter [34].

Dose-response experiments were performed to determine if recruitment of ER α to AHR target promoters occurred at lower doses of PCB126 and E2. As shown in Figure 3, increasing doses of PCB126 resulted in a dose-dependent increase in AHR and ER α recruitment to CYP1A1 and CYP1B1 promoters. AHR was strongly recruited to CYP1A1 and CYP1B1 at 10 nM PCB126 to a level approximately half of that induced by 1 μ M PCB126. A small, but a significant, decrease in the recruitment of AHR was seen for the 10 nM PCB126 + 1 nM E2 or 10 nM E2 co-treatments compared with PCB126 alone. A significant increase in ER α recruitment was observed at 10 nM PCB126, which increased with increasing dose of PCB126. As was observed in Figure 2, co-treatment with E2 increased promoter enrichment of ER α at CYP1A1 and CYP1B1; however, PCB126 co-treatment with 1 nM E2 or 10 nM E2 resulted in a similar level of ER α recruitment to either promoter. Although the doses of PCBs used in the present study exceed environmentally relevant doses, this mechanistic analysis has clearly demonstrated the dose-dependent recruitment of ER α to active AHR. No promoter occupancy of ER α or AHR at CYP1A1 or CYP1B1 was observed with increasing PCB104 treatments (Figure 3).

PCB126, but not PCB104, inhibits E2-dependent pS2 expression

Recent studies have reported that PCB126 and other AHR agonists directly bind and activate ER α [24]. We were therefore interested

Figure 2 PCB126-dependent recruitment of ER α , AHR and ARNT to *CYP1A1* and *CYP1B1* promoters

T-47D cells were treated with 1 μ M PCB or 1 μ M PCB + 10 nM E2 for the indicated amount of time. ChIP assays were performed with primer pairs specific to the XRE enhancer regions of *CYP1A1* or *CYP1B1* by using qRT-PCR as described in the Materials and methods section. Results shown are means $\pm$ S.D. for at least two independent experiments. Fold enrichment levels significantly ($P < 0.05$) greater than zero time controls are indicated by an asterisk, and fold enrichment levels significantly ($P < 0.05$) different between the PCB-treated and PCB + E2-treated time-matched samples are indicated by a dagger.

Figure 3 Dose-dependent recruitment of ER α and AHR to *CYP1A1* and *CYP1B1* promoters

T-47D cells were treated with 0.01, 0.1 or 1 μ M PCB alone or in combination with 1 or 10 nM E2 for 1 h. ChIP assays were performed with primer pairs specific to the XRE enhancer regions of *CYP1A1* or *CYP1B1* by using qRT-PCR as described in the Materials and methods section. Results shown are means $\pm$ S.D. for three independent experiments. Fold enrichment levels significantly ($P < 0.05$) different between the PCB-treated and PCB + E2 samples are indicated by an asterisk.

in examining the ability of PCB126 and PCB104 to inhibit or induce ER α activity and the competitive binding profiles of both compounds to ERs. As expected, co-treatment of TCDD or PCB126 with E2 significantly inhibited E2-induced pS2

expression after 24 h treatment (Figure 4A). PCB126 failed to significantly induce pS2 expression; in contrast, PCB104 significantly induced pS2 expression albeit to a lower level than E2 alone. Co-treatment with PCB104 and E2 failed to inhibit

Figure 4 PCB126 and PCB104 differentially modulate ER-mediated induction of pS2 expression

(A) qRT-PCR analysis of total RNA was isolated from T-47D human breast cancer cells treated with solvent (DMSO), 1 μ M PCB or 1 μ M PCB + 10 nM E2, 10 nM TCDD, 10 nM E2 or 10 nM TCDD + 10 nM E2 and amplified with primers recognizing pS2 mRNA. Results shown are means $\pm$ S.D. for at least two independent experiments. RNA expression levels significantly ($P < 0.05$) greater than zero time or solvent controls are indicated by an asterisk. RNA expression levels significantly ($P < 0.05$) different between the PCB-treated and PCB + E2-treated time-matched samples are indicated by a dagger. (B) Recruitment of ER α to pS2 was determined by ChIP assays and analysed by qRT-PCR as previously described [8].

Table 1 Ability of selected ligands to competitively displace E2 from either ER α LBD

Competition-based ligand binding assay. Purified LBDs of hER α and hER β were incubated with 1.2 nM [3 H]E2 and with between 38 pM and 0.1 mM E2, Genistein, TCDD, PCB126 or PCB104. The IC₅₀ of each ligand is displayed as the mean $\pm$ S.D. for four independent experiments. n.d., Not detected.

Compound	ER α IC ₅₀ (nM)
E2	1.4 $\pm$ 0.4
Genistein	515.8 $\pm$ 52.5
PCB104	n.d.
PCB126	n.d.
TCDD	n.d.

E2-induced expression. These results agree with previous studies describing the oestrogenic activity of PCB104 and the antioestrogenic activity of PCB126 [27,28,35]. ChIP assays showed that co-treatment of PCB126, but not PCB104, with E2 inhibited E2-induced recruitment of ER α to pS2, which was in agreement with the pS2 expression results (Figure 4B). No ER α recruitment to pS2 was observed following treatment with PCB126 or PCB104 alone. In contrast with recent reports [24], neither PCB126 nor PCB104 directly bound to ER α (Table 1). Since the IC₅₀ values for E2 and the phyto-oestrogen Genistein were in agreement with other reports [28,36], the lack of PCB

binding was not due to our assay conditions. The inability of PCB104 to competitively displace E2 from either ER α despite its ability to induce pS2 expression is not surprising since the hydroxylated metabolites of PCB104 are primarily responsible for its oestrogenic effects [27,28].

Effect of SERMs (selective ER modulators) on PCB126 induced expression of CYP1A1 and CYP1B1 mRNA

We then investigated the effects of the SERMs, 4OH-TAM (4-hydroxytamoxifen) and ICI182,780 on PCB126-induced CYP1A1 and CYP1B1 expression and ability to induce recruitment of ER α to CYP1A1 and CYP1B1 gene promoters. Since 4OH-TAM has been shown to preferentially inhibit AF2 activity, whereas ICI182,780 inhibits both AF1 and AF2 function, treatment with these SERMs allows us to determine the relative contribution of AF1 and AF2 to the recruitment of ER α to AHR target genes. Co-treatment of T-47D cells with PCB126 (1 μ M) with either 4OH-TAM (100 nM) or ICI182,780 (100 nM) did not significantly affect CYP1A1 or CYP1B1 mRNA expression following 6 or 24 h exposures compared with PCB126 alone (Figures 5A and 5B). Neither 4OH-TAM nor ICI182,780 alone significantly increased CYP1A1 or CYP1B1 mRNA expression levels. To determine the effect of SERMs on PCB126-dependent recruitment of AHR and ER α to CYP1A1 and CYP1B1, ChIP assays were performed on treated T-47D cells. PCB126-induced AHR recruitment to CYP1A1 and CYP1B1 was unaffected by co-treatment with either 4OH-TAM or ICI182,780. However, co-treatment of PCB126 with either SERM did not enhance ER α promoter occupancy at CYP1A1 or CYP1B1 observed as was observed in PCB126 + E2-treated samples (Figures 5C and 5D).

ER α expression enhances CYP1A1 but not CYP1B1 expression

The effect of TCDD + E2 on TCDD-induced gene expression is controversial and influenced by both cell type and culture conditions [37,38]. Our previous studies and those of others suggest that ER α , rather than E2, influences AHR transactivation [39]. To determine the effects of ER α on TCDD-induced transcription, qRT-PCR of CYP1A1 and CYP1B1 mRNA was performed in ER-negative HEK-293 cells stably expressing ER α with PCBs in the presence or absence of E2 (Figure 6). Since ER α has been shown to interact with AHR through a region in its N-terminal AF1 domain [21], we also analysed cells stably expressing an ER α N-terminally deleted variant lacking its AF1 domain (ER α ΔAF1). Overexpression of each of the ER α wild-type and ER α ΔAF1 variant increased the basal level of CYP1A1 mRNA expression. ER α overexpression resulted in a significant increase in PCB126-induced CYP1A1 expression above that of the empty vector control. No increases in CYP1A1 expression were observed in cells overexpressing the ER α ΔAF1 variant. As was observed for T-47D cells, co-treatment with E2 did not influence PCB126-induced CYP1A1 mRNA expression compared with PCB126 alone. PCB126 treatment resulted in a 2–2.5-fold increase in CYP1B1 expression, which was unaffected by co-treatment with E2. Overexpression of ER α did not significantly affect PCB126-dependent increase in CYP1B1 mRNA expression, but the weak induction of CYP1B1 expression was reduced by ER α ΔAF1 overexpression. PCB104 failed to induce CYP1A1 or CYP1B1 mRNA expression. Expression of ER α and ER α ΔAF1 in the cell lines was verified with Western blot (Figure 6C) using a rabbit polyclonal anti-HC20 antibody directed against ER α C-terminal region. The expected sizes were: ER α wild-type, 66 kDa; and ER α ΔAF1, 47 kDa. A mouse monoclonal anti- β -actin antibody was used as an internal loading control.

Figure 5 Effect of SERM co-treatment on AHR-dependent gene expression and ER α recruitment to *CYP1A1* and *CYP1B1*

T-47D human breast cancer cells were treated with solvent (DMSO), 1 μ M PCB 126 (126) alone or in combination with 100 nM 4OH-TAM, 100 nM ICI182,780 (ICI) or 10 nM E2 for the times indicated. (**A, B**) Total RNA was isolated from T-47D cells treated with 1 μ M PCB or 1 μ M PCB + 10 nM E2, DNase I-treated, and amplified with primers recognizing *CYP1A1* or *CYP1B1* mRNA. Results shown are means $\pm$ S.D. for at least two independent experiments. RNA expression levels significantly ($P < 0.05$) greater than solvent controls are indicated by an asterisk. (**C, D**) ChIP assays were performed on T-47D cells treated for 1 h with solvents or ligands and the resulting DNA was amplified by qRT-PCR using specific primers to the XRE enhancer regions of *CYP1A1* or *CYP1B1*, as described in the Materials and methods section. Results shown are means $\pm$ S.D. for at least two independent experiments. Promoter occupancy levels significantly ($P < 0.05$) greater than DMSO are indicated by an asterisk. Promoter occupancy levels significantly ($P < 0.05$) different between PCB126 and co-treatment with SERMs or E2 are indicated by a dagger.

ChIP assays were performed to determine if differences in *CYP1A1* and *CYP1B1* expression observed in the stable HEK-293 cells were due to differential recruitment of ER α and ER α Δ AF1. Although the AHR interaction region of ER α has been mapped to a short amino-acid motif within ER α AF1 domain [21], robust promoter occupancy of both ER α and ER α Δ AF1 was observed at *CYP1A1* and *CYP1B1* (Figure 7). Significant promoter binding of ER α to *CYP1B1*, but not *CYP1A1*, was observed in the absence of PCB126. In contrast, ligand-independent recruitment of ER α Δ AF1 was observed at both promoters. Ligand-independent promoter binding was determined by comparing the level of occupancy of ER α or ER α Δ AF1 with that of IgG for the DMSO-treated cells. As observed in the breast cancer cells, addition of PCB126 resulted in an increase in the recruitment of ER α to both promoters, but in contrast with the T-47D cells, co-treatment with E2 had no effect (Figure 7). A small increase in ER α Δ AF1 was observed at *CYP1B1*, but not at *CYP1A1*. PCB126-induced recruitment of AHR was not significantly influenced by ER α or ER α Δ AF1 or by E2 co-treatment. The lack of increase in promoter occupancy of ER α and ER α Δ AF1 supports the findings of the following E2 co-treatment in influencing *CYP1A1* and *CYP1B1* expression levels (Figure 6). The recruitment of ER α and ER α Δ AF1 observed in the stable HEK-293 cells might be cell-type-specific or the result of the

overexpression of ER α and ER α Δ AF1 in these stable cells lines, which is at approx. 80 times the levels of wild-type ER α found in T-47D cells (J. Matthews and L. MacPherson, unpublished work). Nonetheless, our results suggest that direct interaction between ER α and AHR is not required to induce recruitment of ER α to AHR target genes.

ER α knockdown inhibits AHR transactivation in mouse mammary cells

To further study the effect of ER α on AHR transactivation and to determine if ER α modulation of AHR transactivation occurs in other species, we used shRNA directed against ER α in HC11 mouse mammary epithelial cells [40]. Stably transfected HC11 cell lines expressing shER α (shRNA targeting ER α) and HC11 cells transfected with empty vector (vector) were treated with PCBs and *Cyp1a1* and *Cyp1b1* mRNA levels examined by qRT-PCR. Stable knockdown of ER α resulted in a significant reduction of, but did not eliminate, the ligand-induced expression of *Cyp1a1* mRNA (Figure 8), supporting our findings with the stable ER α -overexpressing HEK-293 cells. *Cyp1b1* expression was strongly induced by PCB126 but not PCB104. Unlike for *Cyp1a1* regulation, knockdown of ER α did not affect PCB126-induced *Cyp1b1* expression. Co-treatment of

Figure 6 Effect of ERα and ERαΔAF1 on PCB-induced CYP1A1 and CYP1B1 mRNA expression

(A, B) Total RNA was isolated from stable FRT, ERα and ERαΔAF1 cells as described in the legend to Figure 1 and the expression levels of CYP1A1 or CYP1B1 were analysed by real-time PCR. Results shown are means $\pm$ S.D. for three independent experiments. RNA expression levels significantly ($P < 0.05$) greater than FRT DMSO are indicated by asterisks. RNA expression levels significantly ($P < 0.05$) different between the treatment-matched FRT and the individual cell lines are indicated by a dagger. (C) Western-blot analysis of ERα and ERαΔAF1 expression in the HEK-293 stable cell lines.

PCB126 + E2 had no effect on Cyp1a1 or Cyp1b1 induction compared with PCB126 alone. Surprisingly, Cyp1b1 expression was induced by PCB104 with ERα knockdown, but not in control cells. In contrast, PCB104 + E2 increased Cyp1b1 expression independently of ERα knockdown. Collectively, these results demonstrate an important role for ERα in modulating AHR transactivation.

DISCUSSION

In the present study, we show that the AHR agonist PCB126, but not PCB104 effectively recruits ERα to CYP1A1 and CYP1B1 promoters, supporting the notion that active AHR is required to induce recruitment of ERα to AHR target gene promoters. Similar to our previous studies with TCDD, we observed maximum recruitment of AHR and ERα to both CYP1A1 and CYP1B1 after approx. 1 h of treatment. Despite differences in PCB126-induced expression levels of CYP1A1 and CYP1B1, comparable levels of promoter occupancy by AHR and ARNT were observed at both promoters. ERα exhibited a PCB126 dose-dependent recruitment to CYP1A1 and CYP1B1 that was increased by co-treatment with E2. Interestingly, the effects of E2 co-treatment were more prevalent at the CYP1B1 promoter. Although this may be due to differences in PCR amplification between CYP1A1 and

CYP1B1, it may also be due to a functional ERE in its proximal promoter region of CYP1B1 [34]. It should be noted that the level of E2-induced ERα binding to CYP1B1 was significantly less than that observed for PCB126 or PCB126 + E2 treatments. PCB104, a non-co-planar and oestrogen-like compound, did not induce AHR-dependent gene expression nor did it cause the recruitment of ERα to AHR target genes, which is in agreement with our previous findings for E2 [8].

TCDD and other AHR agonists have been reported to exhibit weak oestrogenic activity in the absence of E2 [41]. PCB126 has also been shown to weakly bind to ERα and has been proposed to directly activate ERα; however, binding was only observed at concentrations higher than 1 μ M [24]. In the present study, neither PCB126 nor TCDD competitively displaced E2 from ERα, which was in agreement with other reports [28]. These differences may be due to assay conditions, since assay set-up can influence the competitive binding profiles of compounds that weakly bind to ERα [28,42]. Our binding assays were performed using the ERα LBD and not full-length receptor, thus we cannot completely exclude the possibility that the reported binding of these compounds may be influenced by other regions of ERα. Nonetheless, since E2 alone does not induce recruitment of ERα to AHR target genes, AHR ligands must activate ER in a manner distinct from E2. Our results suggest that the recruitment of ERα is due to activated AHR and not due to the direct activation of ERα by AHR ligands. AHR agonists activate a number of different signalling cascades including mitogen-activated protein kinases, also known to modulate ERα activity [43,44]; thus it is reasonable to hypothesize that AHR agonists may indirectly activate ERs, causing them to modulate AHR signalling via a non-classical protein–protein interaction mechanism. In support of this notion, the phosphorylation-sensitive ERα AF1 region is important in mediating interaction between AHR and ERα [21]. However, we observed promoter occupancy of ERα and ERαΔAF1 to both CYP1A1 and CYP1B1 in HEK-293 cells stably expressing both receptors. The binding of ERα and ERαΔAF1 to CYP1A1 and CYP1B1 may be influenced by the high level of expression of both receptors, but these results also suggest that the AF1 is not required for recruitment of ERα to AHR target genes. Moreover, co-treatment of T-47D cells with PCB126 and either 4OH-TAM, which preferentially inhibits AF2 activity, or ICI182,780, which inhibits both AF1 and AF2 functions, did not result in an increase in the promoter occupancy of ERα at CYP1A1 and CYP1B1 compared with PCB126 alone. These results suggest that the AF2 activity regulates recruitment of ERα to AHR target genes and that direct interaction between AHR and ERα is not required. Studies are currently underway to investigate cell-type-specificity and to determine the contribution of different ERα functional domains in regulating recruitment of ERα to AHR target genes.

ERα-dependent AHR transactivation is a contentious issue in the field of AHR/ERα cross-talk, since ERα effects on AHR activity vary from activation [8,39], inhibition [22,37] to no effect [38]. Under our assay conditions, co-treatment with E2 had no effect on the AHR agonist-induced expression of endogenous AHR target genes, whereas overexpression and targeted knockdown of ERα significantly enhanced and repressed AHR transactivation respectively. Although an increasing number of studies, including this one, have shown AHR ligand-dependent recruitment of ERα to AHR target gene promoters [8,22,24,45], the physiological relevance of these findings is unknown. ERα modulates pathways mediated by STAT5 (signal transducer and activator of transcription 5) [46], NF-κB (nuclear factor κB) [47] and TNFα (tumour necrosis factor α) [48]. ERα modulation of NF-κB signalling is illustrated by the transrepression of

Figure 7 Recruitment of ER α , ER α Δ AF and AHR to *CYP1A1* and *CYP1B1* promoters in stable HEK-293 cells

HEK-293 cells were treated with 1 μ M PCB alone or in combination with 10 nM E2 for 1 h. ChIP assays were performed with primer pairs specific to the XRE enhancer regions of *CYP1A1* or *CYP1B1* by using qRT-PCR as described in the Materials and methods section. Results shown are means $\pm$ S.D. for at least two independent experiments. Promoter occupancy levels significantly ($P < 0.01$) greater than IgG (antibody control) are indicated by an asterisk. Promoter occupancy levels significantly ($P < 0.05$) different between solvent (DMSO)-treated and PCB126- or PCB126 + E2-treated cells are indicated by a dagger.

Figure 8 Promoter-specific modulation of AHR-dependent transcription by shRNA-mediated ER α knockdown

Total RNA was isolated from HC11 mouse mammary cells treated with solvent (DMSO), 1 μ M PCB or 1 μ M PCB + 10 nM E2, as described in the legend to Figure 1 and amplified with primers recognizing (A) *Cyp1a1* or (B) *Cyp1b1* mRNA as described in the Materials and methods section. (C) ER α expression levels in vector control and shRNA-mediated knockdown of ER α (shER α) cell lines. Results shown are means $\pm$ S.D. for at least three independent experiments. RNA expression levels significantly ($P < 0.05$) different from treatment-matched vector controls are indicated by an asterisk. RNA expression levels significantly ($P < 0.05$) different between the shER α treatment-matched samples are indicated by a dagger. ER α mRNA expression levels significantly ($P < 0.001$) different from vector controls are indicated by double asterisks.

interleukin 6 promoter by liganded ER α . Recent studies have shown that E2-bound ER α inhibits, whereas unliganded ER α activates TNF α transactivation [48], highlighting the importance of the non-traditional regulatory roles of ER α and E2-independent functions of ER α . Interestingly, the effects of ER α on AHR transactivation were observed for the regulation of *Cyp1a1* but not *Cyp1b1*, even though ER α was strongly recruited to both promoters following PCB126 treatment. These promoter-specific differences in the modulation of AHR transactivation by ER α may be the result of recruitment of other co-regulator proteins to these promoters. Alternatively, the effects of ER α on *Cyp1b1* expression may not occur within the 24 h treatment protocol used in our studies, and perhaps longer treatment regions are needed. We have previously suggested that the sequestering of ER α by AHR represents a novel mechanism of inhibitory cross-talk between AHR and ER α [8,9]. In agreement with this notion were our findings that inhibition of ER α -dependent increases in pS2 expression correlated with the recruitment of ER α to *CYP1A1* or *CYP1B1* and its reduced recruitment to the pS2 promoter. Alternatively, recruitment of ER α to *CYP1A1* and *CYP1B1* may be part of a complex regulatory loop in oestrogen signalling where E2 metabolism is regulated by ER α activity. Whether the ER modulation of AHR transactivation or recruitment of ER α to AHR target gene promoters is restricted to a limited set of AHR target genes has not been determined.

Although the importance of ER α in physiology is well established, the physiological role of AHR is still uncertain. The high degree of conservation of AHR suggests an important fundamental role in cellular physiology [49]. Studies of *Ahr*-null mice provide evidence for a role of AHR in mediating the toxicity and deleterious effects of dioxin, in development and in the immune system [50]. *Ahr*-null female mice exhibit reduced fertility and the AHR regulates the expression of ovarian P450 aromatase (*Cyp19*), a key enzyme in E2 synthesis, suggesting that AHR has a key role in reproduction [15,51]. The inhibitory effects of AHR ligands on ER α -dependent activity are well established; however, the recruitment of ER α to transcriptionally active AHR

suggests that these two receptor systems are more functionally related than previously thought. Here we show that co-treatment of E2 with PCB126 increases the promoter occupancy of ER α at AHR target gene promoters, suggesting that the circulating levels of E2 in combination with exposure to AHR active ligands influence the genomic binding patterns of ER α . PCB126 induction of CYP1A1 was increased by expression of ER α and reduced by ER α knockdown, suggesting that recruitment of unliganded ER α is important for AHR transactivation of CYP1A1 but the increased binding of liganded ER α following E2 co-treatment makes no further contribution. We have suggested that the increased recruitment observed following co-treatment with E2 and subsequent receptor dimerization represents a mechanism by which activated AHR inhibits ER α signalling [9]. Since ER α does not exert the same effects on CYP1B1 gene expression, it is still unclear whether ER α is a general or gene-specific modulator of AHR transactivation. Collectively, the reduced reproductive capacity of Ahr-null mice and the well-documented inhibitory effects of TCDD on reproductive success, as well as the recent findings of the presence of ER α at AHR target gene promoters suggest that AHR has an important physiological role in ER α signalling. Despite several studies, we do not fully understand the intricate physiological relationship between these two important signalling pathways. Combining sophisticated genome-wide analysis of transcription factor binding patterns with carefully designed studies in appropriate cell-based and animal models will be important in dissecting the physiological importance of cross-talk between these receptor systems.

In summary, our results demonstrate AHR ligand-dependent activation of ER α causing its recruitment to AHR-regulated gene promoters. Our study supports the notion that risk assessment of human health exposure to AHR ligands must consider that many of these compounds modulate the activity of multiple receptor systems. We also observe that ER α is a promoter-specific modulator of AHR-dependent transcription, highlighting the tight functional link between these two receptor systems.

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REFERENCES

- Denison, M. S. and Nagy, S. R. (2003) Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. *Annu. Rev. Pharmacol. Toxicol.* **43**, 309–334.
- Safe, S. (1993) Toxicology, structure–function relationship, and human and environmental health impacts of polychlorinated biphenyls: progress and problems. *Environ. Health Perspect.* **100**, 259–268.
- Nebert, D. W., Dalton, T. P., Okey, A. B. and Gonzalez, F. J. (2004) Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. *J. Biol. Chem.* **279**, 23847–23850.
- Hankinson, O. (1995) The aryl hydrocarbon receptor complex. *Annu. Rev. Pharmacol. Toxicol.* **35**, 307–340.
- Safe, S. H. (1995) Environmental and dietary estrogens and human health: is there a problem? *Environ. Health Perspect.* **103**, 346–351.
- Klinge, C. M., Kaur, K. and Swanson, H. I. (2000) The aryl hydrocarbon receptor interacts with estrogen receptor α and orphan receptors COUP-TFI and ER α 1. *Arch. Biochem. Biophys.* **373**, 163–174.
- Safe, S. and Wormke, M. (2003) Inhibitory aryl hydrocarbon receptor–estrogen receptor α cross-talk and mechanisms of action. *Chem. Res. Toxicol.* **16**, 807–816.
- Matthews, J., Wihlen, B., Thomsen, J. and Gustafsson, J. A. (2005) Aryl hydrocarbon receptor-mediated transcription: ligand-dependent recruitment of estrogen receptor α to 2,3,7,8-tetrachlorodibenzo-p-dioxin-responsive promoters. *Mol. Cell. Biol.* **25**, 5317–5328.
- Matthews, J. and Gustafsson, J. A. (2006) Estrogen receptor and aryl hydrocarbon receptor signaling pathways. *Nucl. Recept. Signal* **4**, e016.
- Rogers, J. M. and Denison, M. S. (2002) Analysis of the antiestrogenic activity of 2,3,7,8-tetrachlorodibenzo-p-dioxin in human ovarian carcinoma BG-1 cells. *Mol. Pharmacol.* **61**, 1393–1403.
- Krishnan, V., Wang, X. and Safe, S. (1994) Estrogen receptor–Sp1 complexes mediate estrogen-induced cathepsin D gene expression in MCF-7 human breast cancer cells. *J. Biol. Chem.* **269**, 15912–15917.
- Kumar, M. B. and Perdew, G. H. (1999) Nuclear receptor coactivator SRC-1 interacts with the Q-rich subdomain of the Ahr and modulates its transactivation potential. *Gene Expression* **8**, 273–286.
- Kobayashi, A., Numayama-Tsuruta, K., Sogawa, K. and Fujii-Kuriyama, Y. (1997) CBP/p300 functions as a possible transcriptional coactivator of Ah receptor nuclear translocator (Arnt). *J. Biochem. (Tokyo)* **122**, 703–710.
- Wormke, M., Stoner, M., Saville, B., Walker, K., Abdelrahim, M., Burghardt, R. and Safe, S. (2003) The aryl hydrocarbon receptor mediates degradation of estrogen receptor α through activation of proteasomes. *Mol. Cell. Biol.* **23**, 1843–1855.
- Baba, T., Mimura, J., Nakamura, N., Harada, N., Yamamoto, M., Morohashi, K. and Fujii-Kuriyama, Y. (2005) Intrinsic function of the aryl hydrocarbon (dioxin) receptor as a key factor in female reproduction. *Mol. Cell. Biol.* **25**, 10040–10051.
- Evans, R. M. (1988) The steroid and thyroid hormone receptor superfamily. *Science* **240**, 889–895.
- Riby, J. E., Chang, G. H., Firestone, G. L. and Bjeldanes, L. F. (2000) Ligand-independent activation of estrogen receptor function by 3,3'-diindolylmethane in human breast cancer cells. *Biochem. Pharmacol.* **60**, 167–177.
- Kousteni, S., Bellido, T., Plotkin, L. I., O'Brien, C. A., Bodenner, D. L., Han, L., Han, K., DiGregorio, G. B., Katzenellenbogen, J. A., Katzenellenbogen, B. S. et al. (2001) Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. *Cell* **104**, 719–730.
- Paech, K., Webb, P., Kuiper, G. G., Nilsson, S., Gustafsson, J., Kushner, P. J. and Scanlan, T. S. (1997) Differential ligand activation of estrogen receptors ER α and ER β at AP1 sites. *Science* **277**, 1508–1510.
- Saville, B., Wormke, M., Wang, F., Nguyen, T., Enmark, E., Kuiper, G., Gustafsson, J. A. and Safe, S. (2000) Ligand-, cell-, and estrogen receptor subtype (α/β)-dependent activation at GC-rich (Sp1) promoter elements. *J. Biol. Chem.* **275**, 5379–5387.
- Ohtake, F., Takeyama, K., Matsumoto, T., Kitagawa, H., Yamamoto, Y., Nohara, K., Tohyama, C., Krust, A., Mimura, J., Chambon, P. et al. (2003) Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. *Nature* **423**, 545–550.
- Beischlag, T. V. and Perdew, G. H. (2005) ER α -AHR-ARNT protein–protein interactions mediate estradiol-dependent transrepression of dioxin-inducible gene transcription. *J. Biol. Chem.* **280**, 21607–21611.
- Chaffin, C. L., Peterson, R. E. and Hutz, R. J. (1996) In utero and lactational exposure of female Holtzman rats to 2,3,7,8-tetrachlorodibenzo-p-dioxin: modulation of the estrogen signal. *Biol. Reprod.* **55**, 62–67.
- Abdelrahim, M., Ariazi, E., Kim, K., Khan, S., Barhoumi, R., Burghardt, R., Liu, S., Hill, D., Finnell, R., Wlodarczyk, B. et al. (2006) 3-Methylcholanthrene and other aryl hydrocarbon receptor agonists directly activate estrogen receptor α . *Cancer Res.* **66**, 2459–2467.
- Safe, S. H. (1994) Polychlorinated biphenyls (PCBs): environmental impact, biochemical and toxic responses, and implications for risk assessment. *Crit. Rev. Toxicol.* **24**, 87–149.
- Hestermann, E. V., Stegeman, J. J. and Hahn, M. E. (2000) Relative contributions of affinity and intrinsic efficacy to aryl hydrocarbon receptor ligand potency. *Toxicol. Appl. Pharmacol.* **168**, 160–172.
- Fielden, M. R., Chen, I., Chittim, B., Safe, S. H. and Zacharewski, T. R. (1997) Examination of the estrogenicity of 2,4,6,2',6'-pentachlorobiphenyl (PCB 104), its hydroxylated metabolite 2,4,6,2',4',6'-hexachlorobiphenyl (HO-PCB 104), and a further chlorinated derivative, 2,4,6,2',4',6'-hexachlorobiphenyl (PCB 155). *Environ. Health Perspect.* **105**, 1238–1248.
- Matthews, J. and Zacharewski, T. (2000) Differential binding affinities of PCBs, HO-PCBs, and aroclors with recombinant human, rainbow trout (*Oncorhynchus mykiss*), and green anole (*Anolis carolinensis*) estrogen receptors, using a semi-high throughput competitive binding assay. *Toxicol. Sci.* **53**, 326–339.
- Van den Berg, M., Birnbaum, L., Bosveld, A. T., Brunstrom, B., Cook, P., Feeley, M., Giesy, J. P., Hanberg, A., Hasegawa, R., Kennedy, S. W. et al. (1998) Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environ. Health Perspect.* **106**, 775–792.
- Takekuma, M., Saito, K., Ogawa, M., Matsumoto, R. and Kobayashi, S. (2004) Levels of PCDDs, PCDFs and Co-PCBs in human milk in Saitama, Japan, and epidemiological research. *Chemosphere* **54**, 127–135.
- Helguero, L. A., Faulds, M. H., Gustafsson, J. A. and Haldosen, L. A. (2005) Estrogen receptors α (ER α) and β (ER β) differentially regulate proliferation and apoptosis of the normal murine mammary epithelial cell line HC11. *Oncogene* **24**, 6605–6616.

- 32 Hestermann, E. V. and Brown, M. (2003) Agonist and chemopreventative ligands induce differential transcriptional cofactor recruitment by aryl hydrocarbon receptor. *Mol. Cell. Biol.* **23**, 7920–7925
- 33 Metivier, R., Penot, G., Hubner, M. R., Reid, G., Brand, H., Kos, M. and Gannon, F. (2003) Estrogen receptor- α directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. *Cell* **115**, 751–763
- 34 Tsuchiya, Y., Nakajima, M., Kyo, S., Kanaya, T., Inoue, M. and Yokoi, T. (2004) Human CYP1B1 is regulated by estradiol via estrogen receptor. *Cancer Res.* **64**, 3119–3125
- 35 Lind, P. M., Eriksen, E. F., Sahlin, L., Edlund, M. and Orberg, J. (1999) Effects of the antiestrogenic environmental pollutant 3,3',4,4',5-pentachlorobiphenyl (PCB #126) in rat bone and uterus: diverging effects in ovariectomized and intact animals. *Toxicol. Appl. Pharmacol.* **154**, 236–244
- 36 Kuiper, G. G., Lemmen, J. G., Carlsson, B., Corton, J. C., Safe, S. H., van der Saag, P. T., van der Burg, B. and Gustafsson, J. A. (1998) Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor β . *Endocrinology* **139**, 4252–4263
- 37 Kharat, I. and Saatcioglu, F. (1996) Antiestrogenic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin are mediated by direct transcriptional interference with the liganded estrogen receptor. Cross-talk between aryl hydrocarbon- and estrogen-mediated signaling. *J. Biol. Chem.* **271**, 10533–10537
- 38 Hoivik, D., Willett, K., Wilson, C. and Safe, S. (1997) Estrogen does not inhibit 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-mediated effects in MCF-7 and Hepa 1c1c7 cells. *J. Biol. Chem.* **272**, 30270–30274
- 39 Thomsen, J. S., Wang, X., Hines, R. N. and Safe, S. (1994) Restoration of aryl hydrocarbon (Ah) responsiveness in MDA-MB-231 human breast cancer cells by transient expression of the estrogen receptor. *Carcinogenesis* **15**, 933–937
- 40 Faulds, M. H., Olsen, H., Helguero, L. A., Gustafsson, J. A. and Haldosen, L. A. (2004) Estrogen receptor functional activity changes during differentiation of mammary epithelial cells. *Mol. Endocrinol.* **18**, 412–421
- 41 Boverhof, D. R., Kwekel, J. C., Humes, D. G., Burgoon, L. D. and Zacharewski, T. R. (2006) Dioxin induces an estrogen-like, estrogen receptor dependent gene expression response in the murine uterus. *Mol. Pharmacol.* **69**, 1599–1606
- 42 Matthews, J., Celius, T., Halgren, R. and Zacharewski, T. (2000) Differential estrogen receptor binding of estrogenic substances: a species comparison. *J. Steroid Biochem. Mol. Biol.* **74**, 223–234
- 43 Tan, Z., Chang, X., Puga, A. and Xia, Y. (2002) Activation of mitogen-activated protein kinases (MAPKs) by aromatic hydrocarbons: role in the regulation of aryl hydrocarbon receptor (AHR) function. *Biochem. Pharmacol.* **64**, 771–780
- 44 Atanaskova, N., Keshamouni, V. G., Krueger, J. S., Schwartz, J. A., Miller, F. and Reddy, K. B. (2002) MAP kinase/estrogen receptor cross-talk enhances estrogen-mediated signaling and tumor growth but does not confer tamoxifen resistance. *Oncogene* **21**, 4000–4008
- 45 Liu, S., Abdelrahim, M., Khan, S., Ariazi, E., Jordan, V. C. and Safe, S. (2006) Aryl hydrocarbon receptor agonists directly activate estrogen receptor α in MCF-7 breast cancer cells. *Biol. Chem.* **387**, 1209–1213
- 46 Faulds, M. H., Pettersson, K., Gustafsson, J. A. and Haldosen, L. A. (2001) Cross-talk between ERs and signal transducer and activator of transcription 5 is E2 dependent and involves two functionally separate mechanisms. *Mol. Endocrinol.* **15**, 1929–1940
- 47 Ray, P., Ghosh, S. K., Zhang, D. H. and Ray, A. (1997) Repression of interleukin-6 gene expression by 17 β -estradiol: inhibition of the DNA-binding activity of the transcription factors NF-IL6 and NF- κ B by the estrogen receptor. *FEBS Lett.* **409**, 79–85
- 48 Cvoro, A., Tzagarakis-Foster, C., Tatomer, D., Paruthiyil, S., Fox, M. S. and Leitman, D. C. (2006) Distinct roles of unliganded and liganded estrogen receptors in transcriptional repression. *Mol. Cell* **21**, 555–564
- 49 Hahn, M. E. (1998) The aryl hydrocarbon receptor: a comparative perspective. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* **121**, 23–53
- 50 Walisser, J. A., Bunker, M. K., Glover, E., Harstad, E. B. and Bradfield, C. A. (2004) Patent ductus venosus and dioxin resistance in mice harboring a hypomorphic Arnt allele. *J. Biol. Chem.* **279**, 16326–16331
- 51 Abbott, B. D., Schmid, J. E., Pitt, J. A., Buckalew, A. R., Wood, C. R., Held, G. A. and Diliberto, J. J. (1999) Adverse reproductive outcomes in the transgenic Ah receptor-deficient mouse. *Toxicol. Appl. Pharmacol.* **155**, 62–70

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