Estrogen Receptor Subtype– and Promoter-Specific Modulation of Aryl Hydrocarbon Receptor–Dependent Transcription

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Abstract

In this study, we examined the role of estrogen receptors (ER) in aryl hydrocarbon receptor (AHR)–dependent transactivation. Chromatin immunoprecipitation assays showed that AHR agonists differentially induced recruitment of ERα to the AHR target genes *CYP1A1* and *CYP1B1*. Cotreatment with 17β-estradiol significantly increased β-naphthoflavone (BNF)– and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin–induced recruitment of ERα to *CYP1A1*, whereas 3,3′-diindolylmethane induced promoter occupancy of ERα at *CYP1A1* that was unaffected by cotreatment with 17β-estradiol. Cyclical recruitment of AHR and ERα to *CYP1A1* was only observed in cells treated with BNF. Stable and subtype-specific knockdown of ERα or ERβ using shRNA showed that suppression of ERα significantly reduced, whereas knockdown of ERβ significantly enhanced, AHR agonist–induced Cyp1a1 expression in HC11 mouse mammary epithelial cells. AHR agonist–induced Cyp1b1 expression was reduced by ERβ knockdown but unaffected by ERα knockdown. The siRNA-mediated knockdown of ERα in MCF-7 human breast cancer cells did not affect 2,3,7,8-tetrachlorodibenzo-*p*-dioxin–dependent regulation of CYP1A1 and CYP1B1 mRNA expression. In agreement with our *in vitro* findings in the HC11 cells, ERα knockout mice exhibit reduced BNF-dependent induction of Cyp1a1 mRNA. These results establish ligand- and promoter-specific influences on the cyclical recruitment patterns for AHR and show ER species-, subtype-, and promoter-specific modulation of AHR-dependent transcription. (Mol Cancer Res 2009;7(6):977–86)

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Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor and member of the basic-helix-loop-helix PER/ARNT/SIM family (1). AHR binds a wide range of endogenous and xenobiotic compounds, including 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; refs. 2, 3). In the absence of ligand, the AHR is located in the cytoplasm bound to a multichaperone protein complex (4). On ligand binding, the AHR translocates to the nucleus, heterodimerizes with aryl hydrocarbon nuclear translocator (ARNT), and the heterodimer binds to their cognate DNA sequences, termed AHR response elements. The activated AHR/ARNT heterodimer recruits coregulators leading to changes in target gene expression, including the phase I detoxifying monooxygenases cytochrome P4501A1 (CYP1A1) and CYP1B1 (5, 6). Studies of animal models reveal that AHR plays a key role in development, immune function, differentiation, and reproduction (7-11).

Estrogens are an important class of hormones that are involved in many physiologic processes (12). Estrogen action is mediated by estrogen receptor (ER)-α and ERβ, which are members of the nuclear receptor superfamily of transcription factors (13). Both ER subtypes regulate gene expression through two different mechanisms: via direct DNA-binding to estrogen response elements or by protein-protein interactions with other transcription factors (14, 15). ERα and ERβ form heterodimers and these heterodimers can modulate the activities of their respective homodimers (16). ERβ exhibits an antagonistic action on ERα-mediated signaling (17, 18), highlighting the importance of balance between the two ER subtypes in estrogen action (19).

Activation of AHR by TCDD and related compounds modulates a number of endocrine systems (20), perhaps most notably by interfering with estrogen signaling. The molecular basis for this inhibitory crosstalk is unclear, and may be due to a combination of several proposed mechanisms (21-23). The antiestrogenic effects of TCDD have led to the proposition that AHR agonists may have therapeutic potential in the treatment of estrogen-dependent cancers. One of these chemicals is indole-3-carbinol, which is found in cruciferous vegetables (24). In the gastrointestinal tract, indole-3-carbinol conjugates are hydrolyzed to many products including 3,3′-diindolylmethane (DIM; refs. 24, 25), which is a partial agonist for AHR. DIM has been reported not only to inhibit estrogenic responses (26) but also to activate ERα in the absence of 17β-estradiol (E_2) via protein kinase A–mediated phosphorylation (27, 28). Although AHR agonists such as 3-methylcholanthrene have been reported to enhance ER-dependent responses

(29, 30), two independent studies have shown that the estrogenic activity of 3-methylcholanthrene is independent of AHR (31, 32). Moreover, a number of AHR ligands have been shown to directly activate ERα, defining a new class of bifunctional AHR/ERα agonists (33, 34). The physiologic relevance of their estrogenic activity is unclear because in the presence of E_2, these ligands inhibit ER activity (21).

The effects of ERs on AHR-dependent transcription are not well characterized. This is complicated by contradictory reports of the influence of E_2 cotreatment on AHR agonist–induced transcription (35-38). However, ERα and ERβ physically interact with AHR (30, 39, 40), and exogenous expression of unliganded ERα restores AHR-dependent responsiveness in ER-negative breast cancer cells (35). This suggests that ERα has an important role in AHR-dependent transcription. To date, little is known about how ERβ may potentially influence AHR activity. Interestingly, ARNT has recently been shown to be a potent coactivator of ERβ-mediated transcription (41). Our studies and those done by other groups have shown that TCDD induces recruitment of ERα to *CYP1A1* and *CYP1B1*, with the level of ERα promoter occupancy being enhanced by cotreatment with E_2, but E_2 alone has no effect (33, 34, 38). However, the role that ERα plays in the regulation of *CYP1A1* remains unclear (22).

In this study, we investigated the functional consequences of AHR agonist–induced recruitment of ERα to *CYP1A1* and *CYP1B1*. Using stable RNA interference–mediated knockdown of ERs and ERα-null mice, we examined the roles of both ERs in AHR-dependent regulation of CYP1A1 and CYP1B1 mRNA levels. Our data show that unliganded ERα is required for maximal AHR-mediated induction of Cyp1a1 mRNA levels in mouse mammary epithelial cells but not in human breast cancer cells, providing new insight into the cell- and species-specific cross talk between the AHR and ER signaling pathways.

Results

AHR Ligands Induce Differential Recruitment of AHR and ERα to the CYP1A1 and CYP1B1 Enhancers

BNF and DIM have been reported to induce cyclical recruitment of AHR to the *CYP1A1* enhancer in MCF-7 human breast cancer cells (42); however, such oscillatory recruitment has not been observed with TCDD treatment (38, 43). Our laboratory and work by others have shown that TCDD, but not E_2, alone induces recruitment of ERα to *CYP1A1* and *CYP1B1* and that the level of ERα recruitment is increased by cotreatment with E_2 (38). These findings have added a new complexity in the well-established crosstalk between the AHR and ER signaling pathways (21). Therefore, we wanted to determine if other AHR ligands, alone or in combination with E_2, also induced ERα recruitment to well-characterized AHR target genes. To this end, chromatin immunoprecipitation (ChIP) assays were done on T-47D human breast cancer cells treated with TCDD, β-naphthoflavone (BNF), and DIM in the presence or absence of E_2. We have reported that the AHR ligand concentrations used in this study do not competitively displace E_2 from ERα or ERβ (44). All AHR ligands induced recruitment of ERα to *CYP1A1* and *CYP1B1* enhancers (Fig. 1), with the level of *CYP1A1* occupancy by ERα varying among the ligands. The recruitment kinetics induced by TCDD was similar to, but more rapid than, that described for similarly treated MCF-7 cells (38). Recruitment of AHR, ARNT, and ERα peaked after 60 minutes, decreased thereafter, and remained at half-maximal level for the remainder of the time course. TCDD treatment induced a maximal 10-fold promoter enrichment of ERα to *CYP1A1*, which was increased to 20-fold by cotreatment of TCDD + E_2. DIM treatment induced maximal enrichment of AHR, ARNT, and ERα at 60 minutes and maintained promoter occupancy at a lower level throughout the time course. Interestingly, DIM treatment induced significantly higher promoter enrichment of ERα (80-fold) at *CYP1A1*, which was about 8-fold higher than that induced by the other AHR ligands tested. Furthermore, the level of ERα recruitment was not further increased by cotreatment with E_2. In agreement with a previous study (42), BNF treatment induced a well-defined oscillatory recruitment pattern for AHR, ARNT, and ERα to *CYP1A1*, with an initial peak observed at 60 minutes and a second peak appearing after 150 minutes. The cyclical recruitment patterns of AHR, ARNT, and ERα were not influenced by BNF + E_2 cotreatment, with the exception of the increase in promoter enrichment of ERα. A slight oscillatory recruitment pattern for AHR, ARNT, and ERα was also evident following DIM treatment, although the second peak at 165 minutes was substantially reduced compared with the initial peak at 60 minutes. Recruitment patterns of ERα, AHR, and ARNT to *CYP1B1* were similar to those observed to *CYP1A1*, with two notable exceptions: (*a*) the level of ERα recruitment was consistently higher at *CYP1B1*, and (*b*) BNF did not induce oscillatory recruitment pattern of AHR or its associated factors to *CYP1B1* (Fig. 1). These data show ligand- and enhancer-specific oscillatory recruitment in AHR-dependent transcription and reveal that AHR ligands induce differential recruitment of ERα to AHR target promoters.

Liganded ERα Does Not Contribute to AHR-Mediated Transactivation

We then determined whether the observed increases in ERα recruitment levels after cotreatment with AHR ligand and E_2 affected AHR-mediated regulation of CYP1A1 and CYP1B1 expression levels. Quantitative PCR data showed that despite the increased recruitment of ERα after AHR ligand cotreatment with E_2, no significant differences in target gene expression were observed (Fig. 2). However, the higher level of ERα recruitment observed following treatment with DIM alone did correlate with a more potent inhibition of E_2-induced trefoil factor 1 (TFF1 or pS2) mRNA expression levels (Fig. 3).

Unliganded ERα Is Required for Maximal Induction of CYP1A1 in HC11 but not MCF-7 Cells

To further study the differential role of the two ER subtypes on the regulation of Cyp1a1 and Cyp1b1 mRNA levels, we used shRNA in the HC11 mouse mammary epithelial cells, known to express both ERα and ERβ (45, 46). Stably transfected HC11 cell lines expressing shRNA targeting ERα (iERα) or ERβ (iERβ) and HC11 cells transfected with empty vector were treated with AHR ligands, and Cyp1a1 and Cyp1b1 expression levels examined by quantitative real-time PCR (Fig. 4). Knockdown of ERα resulted in a significant

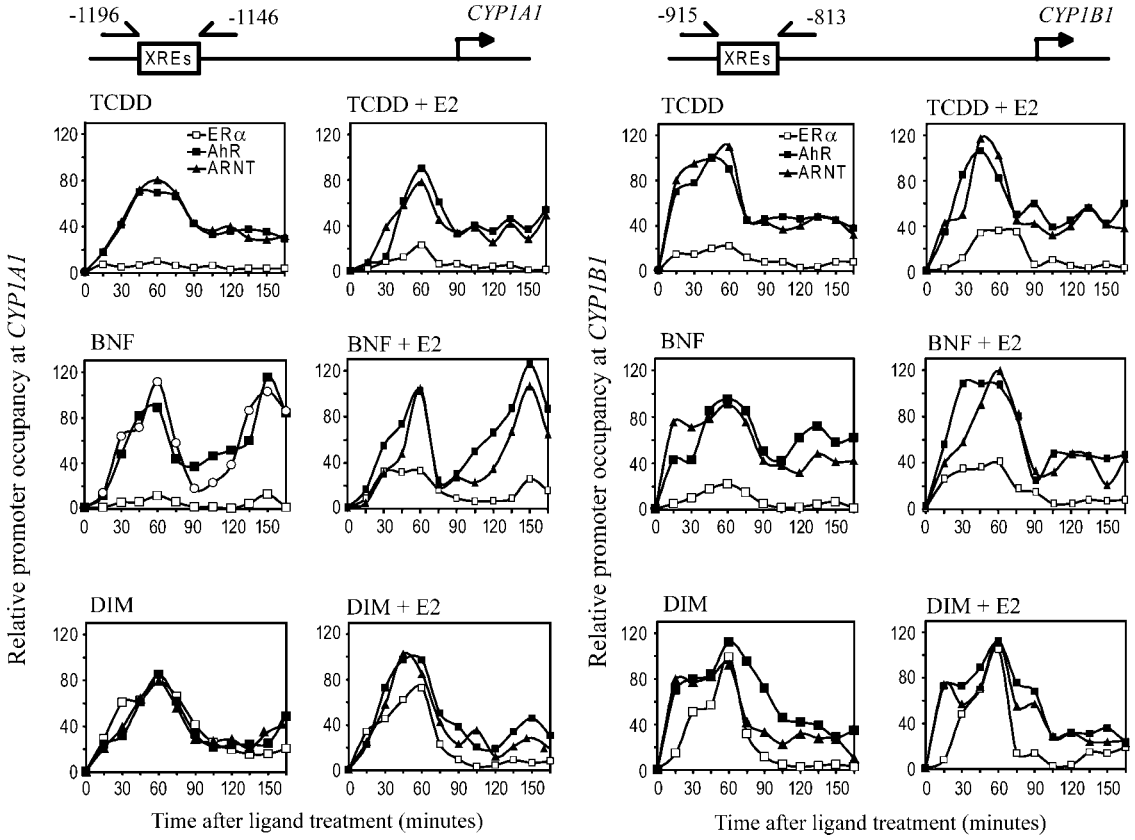

FIGURE 1. Ligand-dependent recruitment of AHR, ARNT, and ERα to the *CYP1A1* and *CYP1B1* enhancer elements. T-47D human breast carcinoma cells were treated with 10 nmol/L TCDD, 1 μmol/L BNF, or 10 μmol/L DIM in the presence or absence of 10 nmol/L E_2 for the indicated time period. ChIP assays were done, as described in Materials and Methods, with antibodies against the indicated proteins. Purified chromatin was analyzed by quantitative real-time PCR and the results are presented as fold enrichment relative to time 0 (no ligand).

reduction of AHR ligand–induced expression of Cyp1a1 mRNA. TCDD-induced Cyp1b1 expression levels in the iERα cells displayed a slight but statistically significant reduction compared with empty vector controls, which was not observed with the other AHR ligands alone or in combination with E_2. Knockdown of ERβ resulted in an increase in Cyp1a1 mRNA levels but a decrease in Cyp1b1 mRNA levels. Interestingly, DIM failed to induce Cyp1a1 gene expression in this cell model, although significant increases in Cyp1b1 expression levels were observed in DIM-treated iERα cells. Time course studies showed that knockdown of ERα caused a reduction in TCDD-dependent Cyp1a1 mRNA expression after 60 minutes of treatment compared with vector controls and iERβ (Fig. 5A). No change in TCDD-dependent Cyp1b1 mRNA expression was observed, with the exception of an increase at the 1-hour time point in iERβ-treated cells (Fig. 5B). ChIP assays revealed an ~2-fold reduction in AHR recruitment to *Cyp1a1* in iERα cells treated with TCDD for 1 and 2 hours (Fig. 5C). No reduction in recruitment of AHR to *Cyp1b1* was observed after 1-hour treatment with TCDD, whereas significant reduction of AHR binding was observed in both the iERα and iERβ cells after 2 hours of treatment with TCDD (Fig. 5D).

To determine the specificity of the results of ERα knockdown in mouse mammary epithelial cells and whether this response is observed in human breast cancer cells, we performed siRNA-mediated knockdown of ERα and AHR in MCF-7 human breast cancer cells and determined the induction of CYP1A1 and CYP1B1 mRNA levels by TCDD. In contrast to the inhibition of AHR-mediated induction of Cyp1a1 expression in HC11 cells stably expressing shRNA directed against ERα, knockdown of ERα in MCF-7 did not affect TCDD-dependent induction of CYP1A1 or CYP1B1 mRNA expression levels (Fig. 6). These data are in agreement with previous reports where the authors showed that knockdown of ERα did not affect AHR ligand–dependent induction of reporter gene activity (33, 34, 38).

Because our *in vitro* data suggested a role for ERα in the regulation of Cyp1a1 in the mouse HC11 cells, we used ERα knockout (ERKO) mice to investigate the role of ERα *in vivo*. Female wild-type and ERKO mice were ovariectomized to avoid any confounding effects from endogenous estrogens. Mice were then treated with 10 mg/kg BNF and assayed for liver Cyp1a1/Cyp1b1 mRNA accumulation. The expression levels of Cyp1a1 mRNA were modestly, but significantly, reduced in the livers of ERKO mice (Fig. 7).

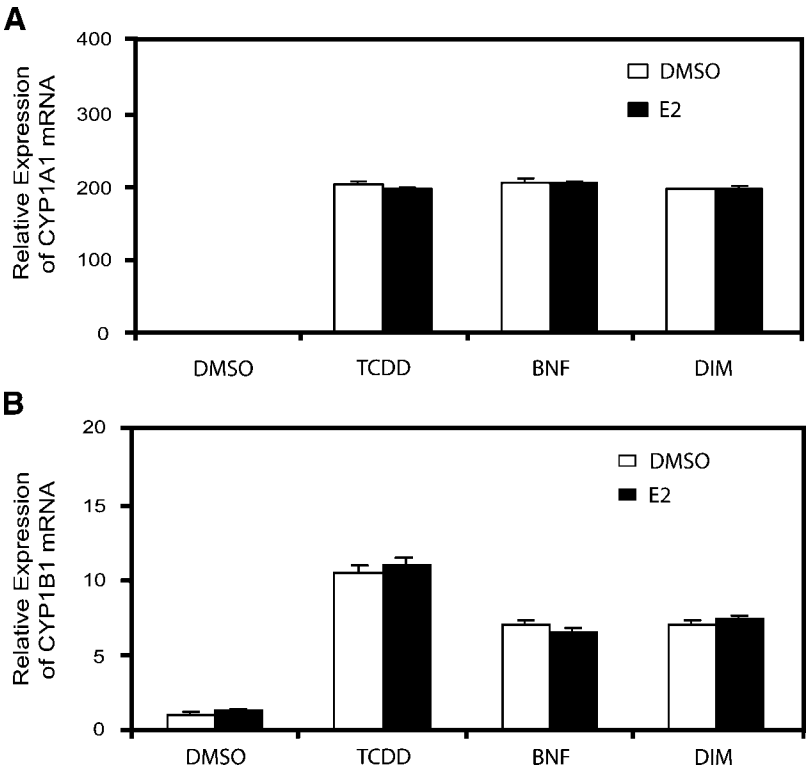

FIGURE 2. Induction of CYP1A1 and CYP1B1 mRNA expression levels by AHR agonists. T-47D cells were treated with 10 nmol/L TCDD, 1 μmol/L BNF, or 10 μmol/L DIM for 24 h. RNA was isolated, reverse transcribed, and quantitative real-time PCR was done as described in Materials and Methods. Results were normalized to expression of 18S rRNA and presented as fold induction of mRNA relative to solvent control (DMSO).

No significant difference in BNF-dependent induction of Cyp1b1 mRNA levels was observed between wild-type and ERKO mice.

Discussion

A renewed interest in AHR/ER cross talk has arisen due to the intriguing findings that AHR and ERα are reciprocally recruited to AHR and ER *cis*-regulatory elements in an AHR agonist–dependent manner (30, 33, 34, 36, 38). The effect of ERα on the AHR signaling pathway is controversial because it is influenced by cell culture conditions and exhibits cell and species specificity (35-38, 47-52). The majority of studies have focused on AHR/ERα cross talk, with few reports investigating the possible interplay between AHR and ERβ. We were therefore interested in determining the ability of other AHR ligands to recruit ERs to AHR target genes and determine the role of each ER subtype in mediating AHR-dependent regulation of CYP1A1 and CYP1B1.

All AHR ligands tested induced ERα binding to the enhancer regions of *CYP1A1* and *CYP1B1*. The magnitude of ERα binding to both regulatory regions was increased with E_2 cotreatment. However, in contrast to the other ligands examined, DIM induced strong ERα recruitment to both *CYP1A1* and *CYP1B1*, which was unaffected by cotreatment with E_2. At the dose used in the present study, DIM activates AHR (53) and also indirectly activates ERα via induction of protein phosphorylation pathways (28). The bifunctional properties of DIM

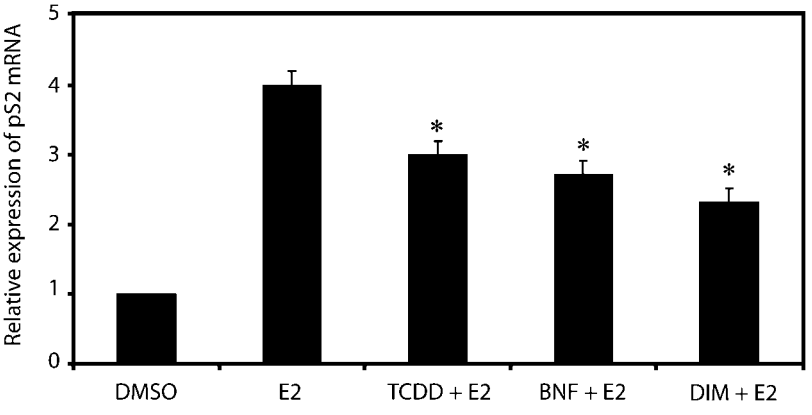

FIGURE 3. Inhibition of E_2 induced pS2 transcription by AHR ligands. T-47D cells were treated with 10 nmol/L E_2 in the presence or absence of 10 nmol/L TCDD, 1 μmol/L BNF, or 10 μmol/L DIM for 24 h. RNA was isolated, reverse transcribed, and quantitative real-time PCR was done as described in Materials and Methods. Results were normalized to expression of 18S rRNA and presented as fold induction of mRNA relative to solvent control (DMSO). *, $P < 0.01$, versus E_2.

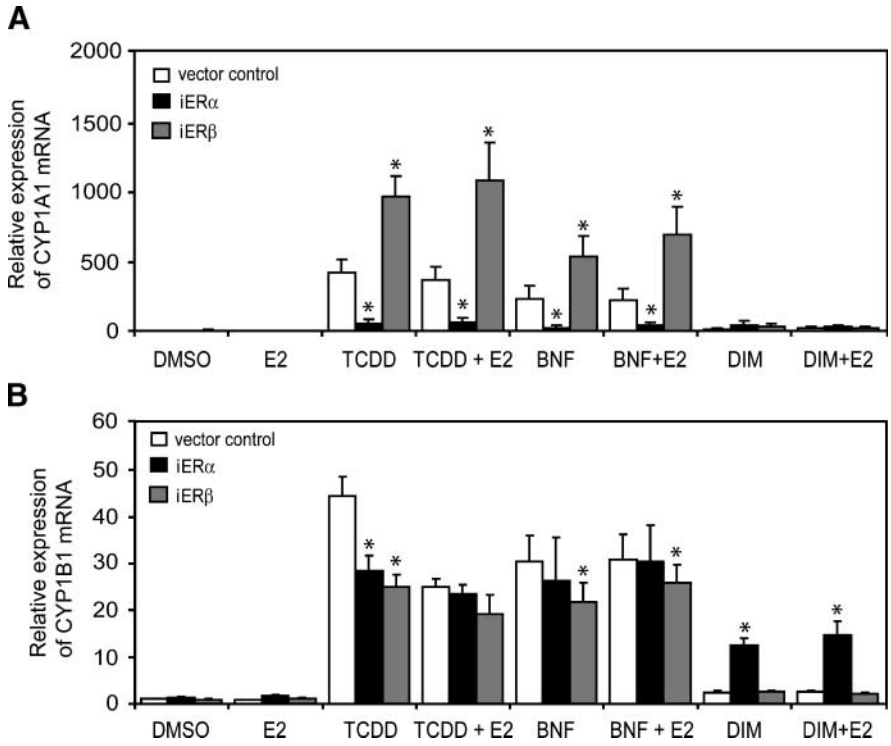

FIGURE 4. ERα or ERβ knockdown differentially modulates AHR-dependent transcription in HC11 cells. HC11 mouse mammary epithelial cells stably expressing shRNA targeted against ERα, ERβ, or vector control were treated with 10 nmol/L TCDD, 1 μmol/L BNF, 10 μmol/L DIM, or solvent control (DMSO) in the presence or absence of 10 nmol/L E_2 for 24 h, after which mRNA was extracted and analyzed by quantitative real-time PCR for CYP1A1 (**A**) and CYP1B1 (**B**). *, $P < 0.05$, between ER-knockdown and vector control.

could explain the notable increase in ERα recruitment observed in this study and by others (34).

Oscillatory recruitment of transcription factors and coregulators to targeted *cis*-regulatory elements is a hallmark of nuclear receptor–mediated transcription (54, 55). It is unclear whether such on and off binding of AHR and its associated factors to chromatin occurs in AHR-mediated transcription (43). In support of the notion that oscillatory recruitment is influenced by the nature of the ligand, we only observed a cyclic recruitment of AHR, ARNT, and ERα to *CYP1A1* in cells treated with BNF and, to a lesser extent, in cells treated with DIM. Other AHR ligands might induce slower oscillatory recruitment patterns for AHR that were not detected within the time frame of our experiments. An extended kinetic analysis is currently under way to investigate this hypothesis. No oscillatory recruitment of AHR, ARNT, or ERα was observed at the CYP1B1 enhancer within the span of our time course, suggesting that recruitment kinetics observed from a transcription factor at one regulatory region may not necessarily occur at other regions regulated by the same factor.

ERα and ERβ exhibit distinct cell and tissue expression patterns (56, 57); however, both ERs are expressed in primary human breast cancers and their coexpression is associated with low biological aggressiveness of breast tumors (58). ERs regulate transcription through direct interactions with estrogen response elements or through an interaction of ER with other transcription factors (59). When both ERs are coexpressed, ERβ inhibits many, but not all, ERα-mediated signaling (17, 19, 60, 61). The inhibitory action of ERβ is due to a combination of a reduction in ERα protein level and reduced recruitment of the activating protein-1 complex (62). Our findings indicate that ERα, but not ERβ, stimulates AHR-mediated transcription of Cyp1a1 in the HC11 mouse mammary cell line but not in MCF-7 human breast cancer cells. The differences between the results of shRNA-mediated knockdown of ERα in HC11 cells and siRNA-mediated knockdown of ERα in MCF-7 cells may be due to a number of possibilities: species differences in AHR and ERα activity between mouse and human; HC11 cells express stable knockdown of ERα whereas ERα levels were transiently knocked down in MCF-7; and/or HC11 cells are nontumorigenic whereas MCF-7 cells are breast cancer cells. Knockdown of ERα or ERβ caused modest reduction of TCDD-induced Cyp1b1 mRNA expression in HC11 cells, but no change in MCF-7 cells. The reduction of TCDD-dependent Cyp1a1 responsiveness in the iERα cells might be the consequence of an alteration in the ERα/ERβ ratio in the HC11 cells and the loss of the inhibitory effects of ERβ on ERα activity. CYP1A1 and CYP1B1 enzymes are important extrahepatic metabolizers of E_2, forming 2-hydroxyestradiol and 4-hydroxyestradiol, respectively. 2-Hydroxyestradiol is the predominant isomer, but the 4-hydroxyestradiol isomer is expressed at abnormally high levels in human breast tumors (63). Moreover, 4-hydroxyestradiol metabolites are carcinogenic in ani-mal models, whereas 2-hydroxyestradiol metabolites are not (64, 65).

Mice lacking ERα showed reduced AHR ligand–dependent induction of Cyp1a1, but not Cyp1b1, mRNA expression. Singhal and colleagues have shown an increased recruitment of ERα to the 5′ regulatory regions of *Ahr* and *Cyp1a1* in liver tissue isolated from mice cotreated with 7,12-dimethylbenz(*a*)anthracene (DMBA) + E_2 compared with DMBA alone (52). Significant increases in Ahr and Cyp1a1 mRNA expression

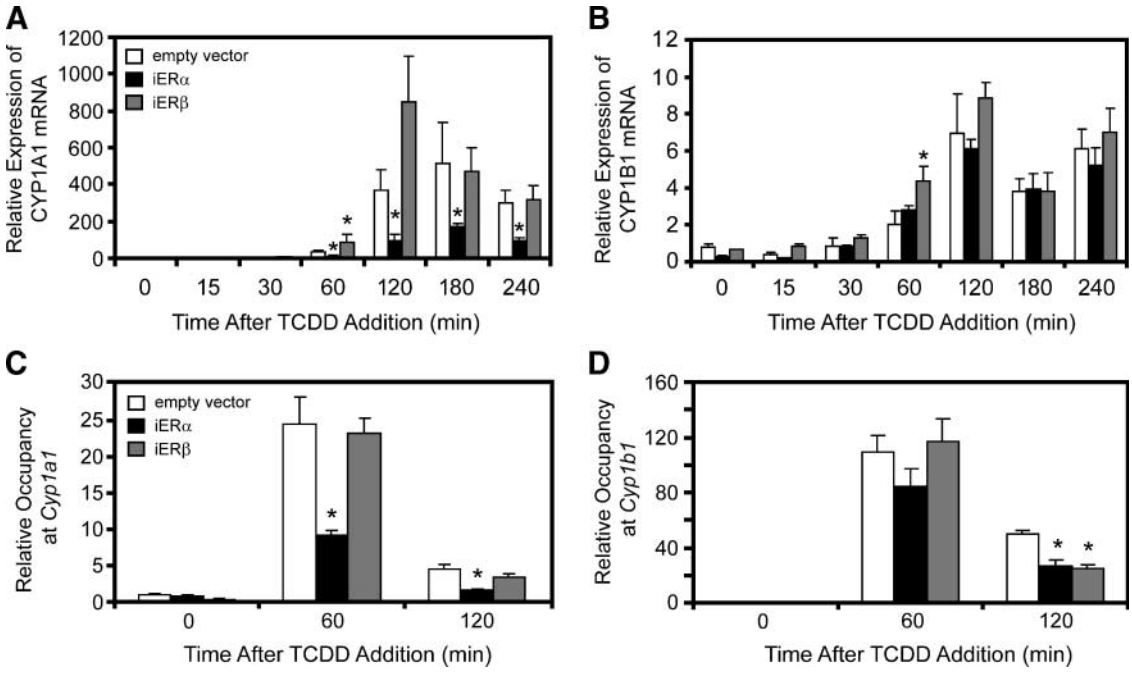

FIGURE 5. Effect of ERα or ERβ knockdown on AHR-dependent transcription in HC11 cells. **A** and **B.** HC11 mouse mammary epithelial cells stably expressing shRNA targeted against ERα, ERβ, or vector control were treated with 10 nmol/L TCDD for the times indicated. RNA was extracted and analyzed with quantitative real-time PCR to detect mRNA expression levels of CYP1A1 (**A**) and CYP1B1 (**B**). **C** and **D.** ChIP analysis of TCDD-dependent recruitment of ER to the *Cyp1a1* (**C**) and *Cyp1b1* (**D**) promoter regions in HC11 vector controls, iERα, or iERβ cells. Cells were treated with 10 nmol/L TCDD for the indicated time periods and ChIP assays were done. *, $P < 0.05$, between ER-knockdown and vector control (**A** and **B**) and compared with time-matched vector control samples (**C** and **D**).

levels in mice treated with DMBA + E_2 versus DMBA alone were also observed (52). These findings show a role for ERα in AHR signaling in mouse liver and provide evidence for a positive role of ERα in AHR-regulated genes other than *Cyp1a1* and *Cyp1b1*. However, the ERα regulation of AHR mRNA expression levels suggests that a mechanism for reduced Cyp1a1 induction may be due to alterations in AHR expression observed in the ERKO. Moreover, Cyp1a1 mRNA exhibits circadian expression in the suprachiasmatic nucleus and liver (66), whereas E_2 has been reported to alter the circadian expression of period (PER) in the liver as well as in the uterus (67, 68). Alterations in the circadian expression of Cyp1a1 could shift the dose-response curve for Cyp1a1 induction by AHR ligands but not necessarily decrease fold induction. Thus, we cannot rule out that the reduced responsiveness of Cyp1a1 to BNF treatment might be due to indirect effects of ERα on AHR-dependent gene expression and Cyp1a1 induction, and not necessarily due to reduced ERα recruitment to *Cyp1a1*.

ERα acts as a dual coactivator/corepressor in nuclear factor-κB–induced activation of tumor necrosis factor α (69), which is determined by its ligand status. However, in agreement with other reports, cotreatment with E_2 increased the recruitment of ERα to *CYP1A1* and *CYP1B1*, without having an effect on the transcription of those genes (33, 38). This suggests either that the increased recruitment of ERα is not necessary for stimulation of CYP1A1 transcription, or that liganded ERα does not affect AHR-mediated transactivation. Despite the relatively high ERα occupancy at *CYP1B1*, no effect on the regulation of CYP1B1 mRNA levels was observed. This was surprising because ERα has been reported to directly regulate CYP1B1 mRNA levels via a half-site estrogen response element in its proximal promoter region (70). We, however, did not observe E_2-dependent increases in CYP1B1 mRNA expression levels in our study. The AHR ligand and E_2 cotreatment–dependent increases in promoter occupancy levels of ERα might represent another mechanism for AHR-dependent inhibition of ERα activity in which ERα is diverted from activating estrogen target genes through facilitated recruitment to AHR target genes. TCDD-dependent inhibition of ERα activity can be observed as soon as after 30 minutes of treatment (71). AHR has been shown to target ERα for degradation by the proteasome complex (23, 40), and recruitment of ERα by activated AHR might represent a mechanism for the proteolytic regulation of ERα levels.

ARNT has been reported to be a potent ERβ coactivator (41), suggesting that AHR/ERβ cross talk may occur through a different mechanism than AHR/ERα cross talk. These findings uncover a potentially new mechanism of AHR-dependent antagonism of ERβ signaling through squelching for limited pools of ARNT. Because the inactivation of the ARNT gene is lethal *in utero* at GD10.5-11 (72, 73), characterization of tissue-specific knockdown (74) or hypomorphic ARNT (75, 76) mouse lines will be important steps to further understand these *in vitro* findings. We also observed distinct differences in the ability of ERs to modulate AHR-dependent regulation of CYP1A1 mRNA levels. Activation of AHR has been reported to induce

recruitment of ERα to ER target genes in the absence of E_2 via direct interactions of AF1 domains of ERα and ERβ with AHR (30). The AF1, however, is not necessary for TCDD-dependent recruitment of ERα to *CYP1A1* or *CYP1B1* (44), suggesting that the mechanism contributing to the recruitment of ERα to AHR-regulated genes is distinct from the recruitment of AHR to ER-regulated genes.

In summary, we have shown that ERα can modulate AHR-mediated transcription of CYP1A1 expression levels *in vitro* and *in vivo*. We also provide evidence for ER subtype–, species-, and cell type–specific modulation of AHR signaling. Future experiments using genome-wide microarrays and the appropriate genetically modified mouse models will be important to further delineate the mechanisms and identify classes of target genes influenced by crosstalk between these two important signaling systems.

Materials and Methods

Reagents

The antibodies used in this study were, for ERα, HC-20; AHR, H-211; and ARNT1, H-172 (all from Santa Cruz Biotechnology). TCDD was purchased from Accustandard. DMSO, BNF, and E_2 were purchased from Sigma. DIM was purchased from BioMol. All ligands were dissolved in DMSO. Cell culture media, media supplements, and fetal bovine serum (FBS) were purchased from Invitrogen. All other chemicals and biochemicals were of the highest quality available from commercial sources.

Cell Culture

T-47D (HTB-133) and MCF-7 (HTB-human breast carcinoma cells) were purchased from American Type Culture Collection and cultured in a 1:1 phenol red–free DMEM and Ham's F-12 nutrient mixture supplemented with 10% FBS. HC11 mouse mammary epithelial cells were cultured in RPMI 1640 supplemented with 10% FBS, 5 μg/mL insulin, 10 ng/mL epidermal growth factor, and 10 μg/mL blasticidin S. All media were supplemented with 2 mmol/L L-glutamine and 1% penicillin/streptomycin, and all cells were maintained at 37°C in 5% CO_2.

RNA Isolation and Quantitative Real-time PCR

T-47D cells were seeded in six-well plates and grown in a 1:1 phenol red–free DMEM and Ham's F-12 nutrient mixture

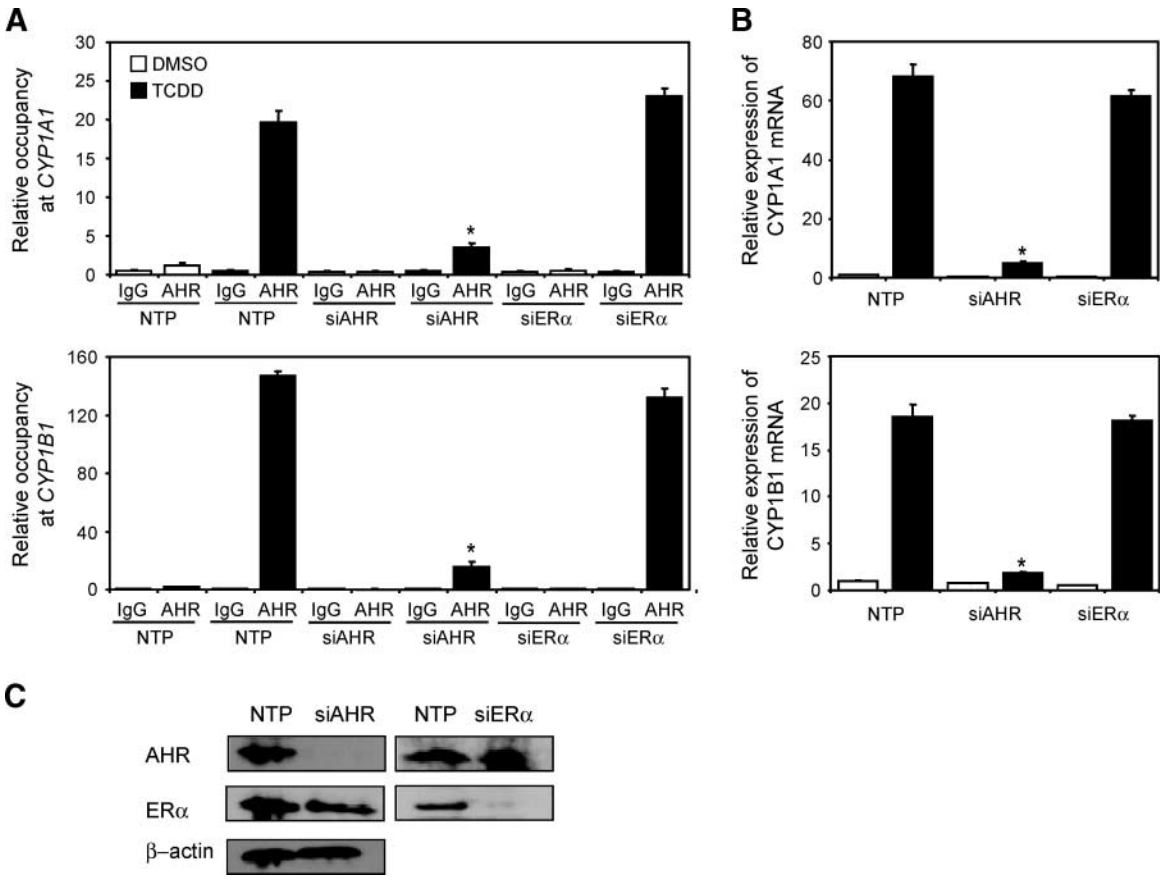

FIGURE 6. Effect of ERα and AHR knockdown on AHR recruitment and AHR-dependent transcription in MCF-7 cells. MCF-7 cells were transfected for 48 h with siRNA and then treated for 1 h with TCDD. **A.** ChIP assays were done with the indicated antibodies, and promoter occupancy for AHR was determined at *CYP1A1* and *CYP1B1* by quantitative real-time PCR. **B.** Analysis of CYP1A1 and CYP1B1 mRNA induction in MCF-7 cells transfected for 48 h with siRNA and then treated for 6 h with TCDD. RNA was isolated and reverse transcribed as described in Materials and Methods and analyzed by quantitative real-time PCR. Data were normalized against time-matched DMSO and to ribosomal 18S levels. **C.** Western blot analysis of AHR and ERα knockdown in MCF-7 cells. β-Actin was used as loading control and each treatment was matched with nontargeting pool siRNA. The data represent the SE of three independent replicates. $P < 0.05$, compared with treatment-matched nontargeting pool (*NTP*) control.

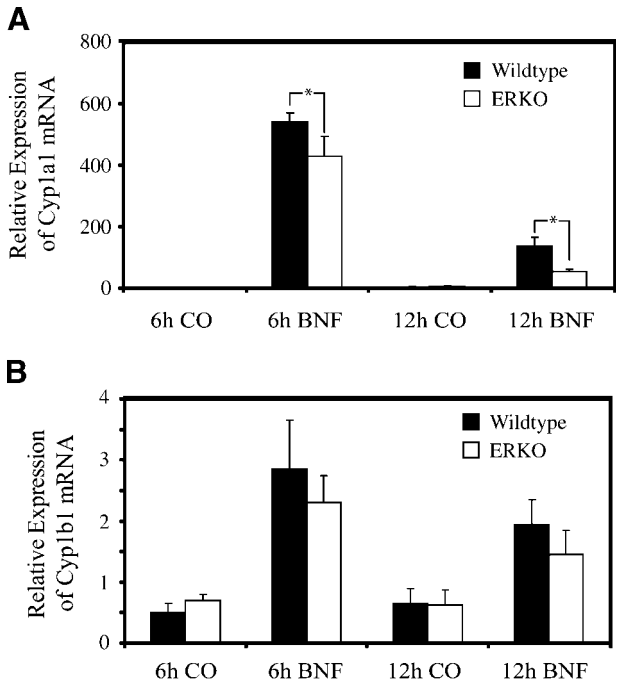

FIGURE 7. Effect of targeted disruption of ERα on AHR-dependent Cyp1a1 and Cyp1b1 expression. Ovariectomized ERα–/– mice (ERKO) and wild-type littermates were given i.p. injections of BNF or solvent control (*CO*) for 6 or 12 h. Livers were harvested and total RNA was extracted, as described in Materials and Methods, and analyzed by quantitative real-time PCR for accumulation of Cyp1a1 (**A**) and Cyp1b1 (**B**) mRNA. *, $P < 0.05$, compared with wild type.

supplemented with 5% dextran-coated charcoal (DCC)-FBS for 24 h. HEK293-ERα, HEK293-ERβ, and HEK293-FRT cells were seeded in six-well plates and grown in phenol red–free, high-glucose DMEM supplemented with 10% DCC-FBS and 0.15 mg/mL hygromycin B. HC11 cells were seeded in six-well plates and grown in phenol red–free RPMI 1640 supplemented with 10% DCC-FBS, 5 μg/mL insulin, 10 ng/mL epidermal growth factor, and 10 μg/mL blasticidin S 24 h before ligand treatment. Cells were then treated with 10 nmol/L E_2, 10 nmol/L TCDD, 10 μmol/L DIM, or 1 μmol/L BNF. For cotreatment studies, 10 nmol/L E_2 was used in combination with the AHR ligand concentration. RNA was isolated using RNeasy spin columns (Qiagen) and 1 μg was reverse transcribed as previously described (38). Quantitative real-time PCR was done using 1 μL of the cDNA synthesis reactions using SYBR Green qPCR Supermix UDG (Invitrogen) or POWER SYBR Green (Applied Biosystems). Results were normalized to expression of 18S rRNA. Primer sequences are available on request.

Chromatin Immunoprecipitation

T-47D cells were seeded in 150-mm dishes and grown for 3 d in a 1:1 phenol red–free DMEM and Ham's F-12 nutrient mixture supplemented with 5% DCC-FBS. HC11 cells were seeded in 150-mm dishes and grown for 3 d in phenol red–free RPMI 1640 supplemented with 10% DCC-FBS, 5 μg/mL insulin, 10 ng/mL epidermal growth factor, and 10 μg/mL blasticidin S. ChIP assays were done as previously described (38) and analyzed by quantitative real-time PCR using SYBR Green qPCR Supermix UDG (Invitrogen) or POWER SYBR Green (Applied Biosystems). Primer sequences are available on request. Results were normalized to time 0 (=1) for each antibody and reported as fold enrichment of the promoter.

Transient Transfection and SiRNA

AHR (L-004990-00-0020) and ERα (L-003401-00-0020) ON-TARGETplus SMART pool siRNA and DharmaFECT1 transfection reagent were purchased from Dharmacon. Briefly, MCF-7 cells were seeded 300,000 per well in a six-well plate containing 2 mL of medium. After 24 h, 2 μmol/L of siRNA against AHR (L-004990-00-0020) or ERα (L-003401-00-0020) or nontargeting pool (D-0011810-10-20; SMARTpool, Dharmacon) was transfected using 4 μL of DharmaFECT and 400 μL of Opti-MEM. The following day, medium was changed using normal plating medium. ChIP assay or mRNA isolation and whole-cell extracts were prepared 48 h after transfection.

Western Blot

Proteins were resolved by SDS/10% PAGE and transferred onto a polyvinylidene difluoride membrane in 25 mmol/L Tris base (pH 8.3) containing 19.2 nmol/L glycine and 20% (v/v) methanol. The membrane was blocked in 2% (w/v) ECL-Advanced blocking agent for 1 h at room temperature with constant rocking and then incubated with 1:1,000 anti-ERα (HC-20, Santa Cruz Biotechnology) or 1:1,000 anti-AHR (SA-210, BioMol) overnight at 4°C with constant rocking. The membrane was then washed thrice in PBS/0.1% Tween and incubated with 1:200,000 horseradish peroxidase–conjugated antirabbit secondary antibody for 1 h at room temperature with constant rocking. After washing, the bands were visualized using ECL-Advanced chemiluminescent substrate (GE Healthcare) according to the manufacturer's instructions. The membranes were exposed to an autoradiography film for 15 s to 2 min. For detection of β-actin, a 1:500,000 dilution of primary mouse anti–β-actin antibody (Sigma) was incubated for 2 h at room temperature followed by a 1-h washing with PBS/Tween before incubation with horseradish peroxidase–conjugated antimouse secondary antibody for 1 h at room temperature. A final 30-min washing in PBS/Tween was done before development with ECL-Advanced chemiluminescent substrate.

Animal Experiments

Animals used for this study were age matched (10-16 mo) ERα−/− mice and wild-type littermates obtained from breeding of heterozygous male and female mice. Mice were housed (12 h light:12 h darkness, at a temperature of 21-22°C, and a relative humidity of 50-62%) in polycarbonate plastic cages (Scanbur) containing wood chips, with free access to fresh water and food pellets, at the infection-free animal facility, Karolinska University Hospital, Huddinge. Female mice were anesthetized with 5 mg/kg midazolam (F. Hoffmann-La Roche Ltd.), 0.1 mg/kg medetomidine (Orion Corp.), and 0.3 mg/kg fentanyl (B. Braun Medical AG), and subsequently bilaterally ovariectomized through a single dorsal incision across the lumbar region, making both ovaries accessible. The ovary-attached fat pads were grasped and pulled out together with the ovary allowing removal of the whole ovary. After a period of recovery, mice were given a single i.p. injection of 10 mg/kg BNF

dissolved in corn oil (Sigma) or vehicle control (corn oil). Mice were sacrificed after 6 and 12 h, and livers were harvested and flash frozen in liquid nitrogen. Approximately 10 μg of liver tissue were excised and RNA was purified using the EZNA total RNA kit (Omega Bio-Tek) according to the manufacturer's instructions. All animal experiments were done at the infection-free animal facility, Karolinska University Hospital, Huddinge in accordance with ethical committee approval.

Statistical Analysis

Statistical comparisons were made using the paired two-tailed Student *t* test where appropriate.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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