ER-alpha-dependent regulation of dioxin inducible gene transcription

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INTRODUCTION

AHR and ER are both ligand activated transcription factors that transduce extra-cellular signals through DNA-binding dependent and independent mechanisms. AHR and the aryl hydrocarbon receptor nuclear translocator (ARNT) form a heterodimeric transcription factor, the aryl hydrocarbon receptor complex (AHRC), that binds a wide variety of environmental pollutants including polycyclic aromatic hydrocarbons (PAH), and halogenated aromatic hydrocarbons (HAH), such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (dioxin, TCDD). The binding of these compounds and subsequent activation of target genes are part of on organism's adaptive response to environmental contaminants.

Like AHR, the estrogen receptor (ER) is a ligand activated transcription factor, which upon ligand binding forms a functional homodimer and binds its cognate response elements, the ERE. As with most NR's, ER is thought to manifest its main biological function by transducing the transcriptional information contained in its response elements. This has been questioned over the past decade by several independent findings. Investigators in Gunther Schutz's laboratory made the startling observation that DNA-binding/dimerization deficient glucocorticoid receptor (GR) mutant mice were viable, while null mutations are lethal[1,2]. These observations demonstrated unequivocally, that the DNA-binding capability of a transcription factor was not necessarily essential for survival and that a classical transcription factor had vital non-DNA binding properties.

The mechanisms by which ER down regulates aromatic hydrocarbon signaling remain unclear. In this study, we demonstrate that a significant component of TCDD-inducible *Cyp1a1* transcription is the result of recruitment of ERα by AHR/ARNT as a transcriptional co-repressor. Both AHR and ARNT were capable of interacting directly with ERα, as ascertained by GST pull-down. Both 17β-estradiol (E2) and tamoxifen repressed TCDD- and PCB126-activated *Cyp1a1* and *Cyp1b1* gene transcription in MCF-7 cells in the presence of cycloheximide, as determined by reverse transcription/real-time PCR. In contrast, E2 had no effect on PAH induced *Cyp1a1* or *Cyp1b1* transcription. Furthermore, ChIP assays have shown that ERα is present at the *Cyp1a1* enhancer only after co-treatment with E2 and TCDD, in MCF-7 cells. Sequential two-step ChIP assays were performed which demonstrate that AHR and ERα are present together at the same time on the *Cyp1a1* enhancer during transrepression. Taken together these data support a role for ER-mediated transrepression of AHR-dependent gene regulation.

Materials and Methods

Reverse Transcription and Real-Time PCR – All experiments were performed as described previously[3]. Cells were maintained in serum-free media lacking Phenol Red for 48 hrs. before treatment with any ligand. Prior to treatment with ligand MCF-7 cells were exposed to cycloheximide (10 μg/ml) for 1 h to halt protein translation. Subsequently, cells where treated either with vehicle (Me_2SO), 2 nM TCDD, 100 nM E2 or a combination of TCDD and E2 for 8 h. Cells were harvested and total RNA was isolated and subjected to reverse transcription. cDNA's were amplified by real-time PCR.

Western Blot Analysis – Whole cell extracts of MCF-7 cells and Western blotting was performed as described previously[4], with minor modifications. After incubation with primary antibodies, blots were incubated with a biotin labeled goat anti-rabbit IgG. Blots were washed and incubated with $I^{[125]}$-labeled strepavidin (Amersham) and exposed to film overnight.

Single and Sequential Two-Step ChIP Assays - Precipitations for ChIP's and re-ChIP's from fixed MCF-7 cell lysates were performed in quadruplicate. One sample from each set was chosen for analysis by single step chromatin immuno-precipitation. For re-ChIP experiments, complexes from the primary ChIP were eluted in 50 μl of

10 mM DTT for 30 min at 37 degrees C, pooled and precipitated with the indicated antibody.

Results and Discussion

ERα Represses TCDD-inducible Transcription - We made the observation that co-transfection of ERα with a *Cyp1a1* promoter driven luciferase vector in Hepa1 cells treated with TCDD with or without E2 leads to E2-mediated repression of TCDD-dependent luciferase activity[5]. In order to assess the direct transcriptional effect of ER-activation on AHRC-dependent gene transcription, we employed reverse-transcription/real-time PCR of the AHRC target genes *Cyp1a1* and *Cyp1b1* in the presence and absence of the protein synthesis inhibitor, cycloheximide. TCDD but not

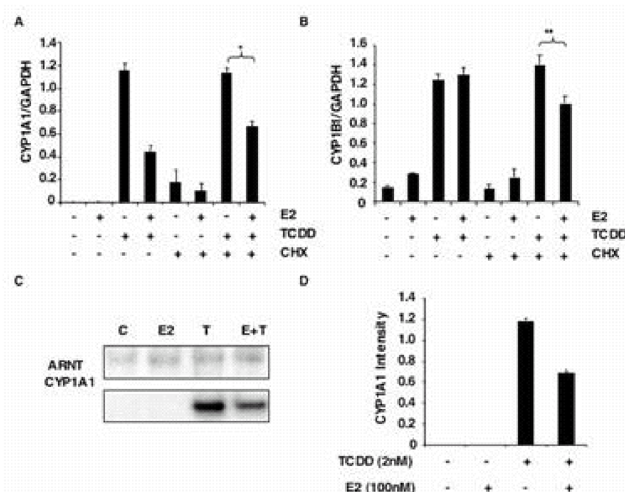

Figure 1. 17β-estradiol represses TCDD inducible endogenous *Cyp1a1* **(A)** and *Cyp1b1* **(B)** transcription in MCF-7 cells. Cells were incubated in the presence or absence of 10 µg/ml cylcoheximide (chx) for 1 h prior to treatment with either E2 (100 nM), or TCDD (2 nM), or both for 7 h. Total RNA was reversed transcribed and *Cyp1a1*, *Cyp1b1*, and *GAPDH* cDNA was amplified by real-time PCR. A paired t-test was performed on the indicated parameters: *p<0.001; **p<0/01. **(C)** MCF-7 cells were treated with Me_2SO (C), 100nM 17β-estradiol (E2), 2 nM TCDD (T), or E2 and TCDD (E+T) together for 18 h. Whole cell extracts were subjected to SDS-PAGE and relative amounts of CYP1A1 and ARNT protein were assessed by Western blot analysis. **(D)** CYP1A1 protein band intensity levels were normalized to that of ARNT and HSP86 with essentially identical results.

100 nM E2 caused a significant increase in *Cyp1a1* gene transcription in MCF-7 cells (Figure 1a). However, E2 (like TCDD, alone) caused a significant increase in *Cyp1b1* gene transcription, consistent with reports that the 5´-regulatory region of the *Cyp1b1* gene harbors an estrogen response element, ERE (45) (Figure 1b). The addition of 100 nM estradiol significantly reduced TCDD induced *Cyp1a1* gene transcription in the presence or absence of cycloheximide (Figure 1a and b) and significantly reduced *Cyp1b1* transcription in the presence of cycloheximide. The observation that maximal *Cyp1a1* gene induction was repressed by approximately 50% in the absence or presence of cycloheximide suggests that both direct transcriptional repression of *Cyp1a1* and not secondary E2 mediated downstream events are responsible for the repressive effects of estrogens on AHRC signaling. Furthermore, the E2-mediated decrease in *Cyp1a1* mRNA production preceded a concomitant decrease in CYP1A1 protein levels (Figure 2c and d) in MCF-7 cells. The observed decrease in corrected CYP1A1 protein levels (approximately 42%) is consistent with the observed decrease in mRNA levels demonstrating that the observed repression of TCDD-inducible transcription by E2 can have an equally profound physiological outcome.

ERα Associates with the *Cyp1a1* Promoter in an AHRC-dependent fashion - We employed the ChIP assay to ascertain the status of ERα at the *Cyp1a1* enhancer in the presence and absence of E2 and TCDD. ERα was greatly enriched at the *Cyp1a1* enhancer only after treatment with a combination of 1 nM TCDD and 100 nM E2 (Figure 2b). As a control, we monitored the presence of AHR, ARNT and ERα over the *pS2* promoter under similar conditions (Figure 2b) with results similar to those observed by other investigators[6]. Furthermore, precipitation of *Cyp1a1* enhancer chromatin was enriched by antibodies directed against the acetylated form (lys 12) of histone H4 (AcH4) in cells treated with TCDD, but this was seemingly reduced by the addition of E2 (figure 2b), suggesting that ERα mediates its repressive effects through a histone de-acetylase dependent mechanism. Chromatin encompassing the pS2 promoter was efficiently precipitated by this antibody under all conditions tested reflecting the high level of *pS2*.

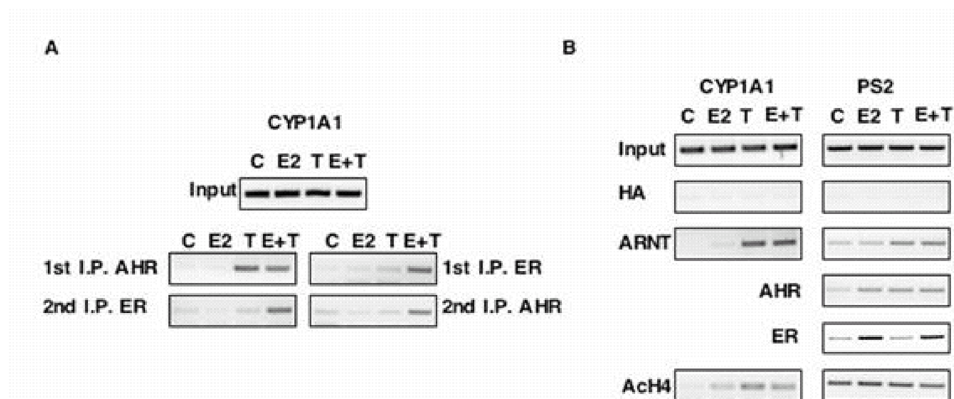

Figure 2. ERα binds the *Cyp1a1* enhancer only in response to a combination of E2 and TCDD. **(A)** Sequential two-step ChIP of AHR and ERα over the *Cyp1a1* enhancer demonstrates that ERα and AHR exist together on the *Cyp1a1* enhancer. **(B)** ChIP analysis of ERα, AHR, ARNT and acetylated histone H4 (AcH4) over the *Cyp1a1* enhancer and *pS2* promoter region. Cells were incubated with either, vehicle (C), 100 nM E2 (E), 1 nM TCDD (T), or E2 + TCDD (E+T).

In order to determine unequivocally if ERα and AHR occupy the same portion of chromatin at the same time we performed sequential precipitations with antibodies for AHR and ERα in MCF-7 cells. After binding to agarose beads, samples were eluted and those precipitated with an anti-AHR were incubated with anti-ERα and those initially precipitated with an anti-ERα were incubated with anti-AHR. Again, complexes were bound to the appropriate secondary Ab-conjugated agarose resin. Both AHR and ERα could be precipitated from ERα and AHR affinity purified samples respectively, on the *Cyp1a1* enhancer (Figure 2a). Thus, we have established that ERα is present at the *Cyp1a1* enhancer only in the presence of both estradiol and TCDD. Furthermore, we have demonstrated by two-step ChIP that AHR and ERα are present at the *Cyp1a1* enhancer at the same time. Taken together, these data strongly suggest that ERα directly interacts with the AHRC multi-protein complex.

Acknowledgments

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