ERα-AHR-ARNT protein-protein interactions mediate estradiol-dependent transrepression of dioxin inducible gene transcription.*

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Running Title: ERα repression of AHR signaling

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The aryl hydrocarbon receptor (AHR) and the aryl hydrocarbon receptor nuclear translocator (ARNT) form a heterodimeric transcription factor upon binding a wide variety of environmental pollutants, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). AHR target gene activation can be repressed by estrogen, and estrogen-like compounds. 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 corepressor. Both AHR and ARNT were capable of interacting directly with ERα, as ascertained by GST pull-down. 17β-estradiol repressed TCDD-activated Cyp1a1 and Cyp1b1 gene transcription in MCF-7 cells in the presence of cycloheximide, as determined by reverse transcription/real-time PCR. 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.

AHR^1 and ER are both ligand activated transcription factors that transduce extracellular signals through DNA-binding dependent and independent mechanisms (1-4). 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) (5), 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 an organism’s adaptive response to environmental contaminants (6). Furthermore, studies in AHR knock-out mice have revealed an important role for AHR in development and physiological homeostasis (7-11).

Unliganded AHR exists in the cytoplasm as part of a multimeric complex containing two molecules of HSP90, the HSP90 co-chaperone p23, and a 36 kDa protein termed hepatitis B virus X-associated protein 2 (XAP2) (12-16). Upon ligand binding, AHR translocates to the nucleus where it associates with ARNT to form a functional transcription factor complex, the AHRC. As an activated complex, the AHRC is capable of recruiting several classes of co-activators such as SRC-1, NCoA2/GRIP1/TIF2 and p/CIP (17,18), RIP140 (19), BRG-1 and components of the mediator complex (20,21), CBP and TRIP230 (22-25). These proteins are incorporated into multimeric complexes, which interact with and modulate the activity of the core transcriptional machinery, as well as modifying local chromatin structure (26). However, the identity and mechanisms whereby ancillary
proteins are recruited by the AHRC to its cognate response element are still, largely, unknown. Elucidation of the molecular mechanisms underlying activated transcription by the AHRC is central to our understanding of development, physiological homeostasis and the complex pathologies responsible for a wide spectrum of human diseases including chemical carcinogenesis, and solid tumor growth.

Like AHR, the estrogen receptor (ER) is a ligand activated transcription factor. ER belongs to the super-family of nuclear hormone receptors (NR) (27), 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 (28) while null mutations are lethal (29). 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 (28). Furthermore, AP-1 regulation of interstitial collagenase was repressed by GR by a direct protein-protein interaction between GR and AP-1 (30-33). GR-mediated transrepression of NF-κB signaling is also well documented (4,34,35). Subsequently, other nuclear hormone receptors, including the thyroid hormone receptor (TR), and ER, were shown to be able to repress AP-1 and NF-κB activity via direct protein-protein interactions (4,34,35). Furthermore, the effects of endocrine disruptors and compounds that mimic the activities of endogenous estrogens affect not only ERE-regulated genes but also genes transrepressed by ER and the relative importance of each phenomenon has yet to be established.

The mechanisms by which PAH/HAH repress ER signaling are well understood and are attributable to direct protein-protein interactions with liganded AHR and ER at the regulatory regions of ER target genes and non-transcriptional or downstream events. However, the mechanisms by which ER down regulates aromatic hydrocarbon signaling remain unclear. Furthermore, the general mechanism in which NR’s tether to other transcription factors and modulate their activities reveals another level of specificity of NR function. In this report, we provide evidence that ERα represses TCDD-inducible Cyp1a1 and Cyp1b1 transcription through direct protein-protein interactions with the AHRC in the regulatory regions of these genes.

**Experimental Procedures**

**Cell Lines and Reagents** - Polyclonal anti-ERα and CYP1A1 antibodies were purchased from Santa Cruz Biotechnology, Inc. Rabbit anti-AHR polyclonal antibody (SA-210) was purchased from Biomol. Affinity purified anti-acetyl histone H4 (Lys12), was purchased from Upstate USA, Inc. Rabbit anti-ARNT polyclonal antibody was the gift of Dr. Oliver Hankinson. HEK-293 and MCF-7 cells were maintained in serum free αMEM lacking phenol red with high glucose, for at least 48 h prior to any treatment or experimental manipulation to ensure that unnecessary activation/down-regulation of ER or other signal transduction pathways would not confound any experimental parameters.

**GST-Pull Down Assays and Transient Transfections and Reporter Gene Assays** – GST-pull down assays were performed as described previously (18). Six well plates containing 293 cells were transfected with 100 ng of the GAL4 UAS reporter pG5E4T, CMV-β-Gal (100 ng) and either 200 ng of pGAL-AHR, or pGAL-ARNT, with or without 200 ng of pG5-ERα. A total of 600 ng of DNA was used for each transfection using empty expression vector when
necessary. Each transfection was achieved using 15 µl of Superfect transfection reagent (Qiagen). Media was changed 3 h after transfection and cells were treated with 100 nM E2. Cells were harvested 16-20 h after transfection and assayed as described previously (17).

Reverse Transcription and Real-Time PCR – Prior to treatment with ligand MCF-7 cells were exposed to cycloheximide (10 µg/ml) for 1 h to halt protein translation. Subsequently, cells were treated either with vehicle (Me2SO), 2 nM TCDD, 100 nM E2 or a combination of TCDD and E2 for 8 h. Cells were harvested in TriZol and total RNA was isolated and subjected to reverse transcription using a High Capacity cDNA Archive kit (Applied Biosystems). cDNA's were amplified by real-time PCR using a DyNAmo HS SYBR Green kit (Finnzymes) according to manufacturer's protocols. Oligonucleotide pairs used to amplify human cDNA sequences were: for Cyp1a1, 5'-TCTTCCTTCGTCCCCCTTAC-3’ and 5’-TGGTTGATCTGCACTTGGT-3’ (forward and reverse, respectively); for Cyp1b1, 5’-CATGGGCTTCTCCAGCTTTGT-3’ and 5’-GGCCACTTCATGGTACGTT-3’; for pS2, 5’-AGTGGCCCGCCGTGAAAG-3’ and 5’-TCTGGAGGGACGTCGATGGT-3’; and for GAPDH 5’-TGCACACCACCACTGCTTAG-3’ and 5’-GGCATGGACTGTCATCGG-3’.

Western Blot Analysis – Whole cell extracts of MCF-7 cells and Western blotting was performed as described previously (17), 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 [35S]-labeled strepavidin (Amersham) and exposed to film overnight. Protein levels were quantified using a Cyclone phosphor imaging system and OptiQuant software.

Single and Sequential Two-Step ChIP Assays - Precipitations for 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-precipitations and these reactions were performed as previously described (23). 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

Pre-natal, peri-natal, as well as long-term exposures to estrogens, xeno-estrogens, and endocrine disruptor's such as TCDD, put individuals at an increased risk of testicular, breast and prostate cancers and developmental defects of the urogenital tract (36,37). Many testicular cancer etiologies implicate the activation of the aryl hydrocarbon receptor (37-39) or conversely, the disruption of the estrogen signaling pathways by these compounds and other endocrines disruptors such as diethylstilbestrol. Subsequent studies continue to re-iterate these findings (40). As a result, cross-talk between AHR and NR pathways have been implicated but studies investigating the molecular mechanisms underlying AHR-NR cross-talk are scarce. Perhaps, one of the most important and intriguing problems concerning the mechanism of NR function, is how they moderate gene transcription in a DNA-binding independent fashion.

AHR and ARNT Interactions with ERα - Experiments employing GST-AHR/ARNT fusions were performed with radiolabeled in vitro translated [35S]-labeled ERα. Consistent with the observations of other investigators (1,2) we were able to demonstrate an interaction between ERα and AHR or ARNT. Furthermore, we were able to demonstrate that the ERα interaction domain within AHR resides within the P/S/T region of AHR's TAD (Figure 1a). GST fusion chimeras of Sp1 and VP16 failed to precipitate in vitro translated ERα.
To test the hypothesis, that ER directly repressed AHRC via AHR’s P/S/T domain, we co-transfected HEK-293 cells with a CYP1A1 promoter-driven luciferase plasmid, pGUDLUC, expression vectors encoding ERα, and wild-type human AHR or a deletion mutant encoding human AHR deleted for it’s P/S/T domain. Cells were treated with vehicle or E2, or E2 in combination with TCDD overnight. Both wild type AHR and AHRΔP/S/T were equally capable of activating transcription from pCYP-GudLuc in a TCDD-dependent fashion (Figure 1b). However, co-transfection of ERα in the presence of 100 nM E2 significantly diminished this activation in each instance (Figure 1b).

In order to determine if ERα can repress either AHR, or ARNT transactivation function, HEK-293 cells were co-transfected with expression cDNA plasmids encoding either GAL4-DNA-binding-domain-AHR carboxy-terminus or full-length ARNT and ERα the GAL4 driven luciferase vector, pG5E4T (Figure 1c). Activated ERα enhanced the GAL4DBD-AHR chimera harboring the P/S/T putative ERα interaction domain, transactivation function significantly (Figure 1d). However, transcriptional activity driven by the GAL4-ARNT chimera was significantly repressed. This suggests that ERα transduces its repressor function via its interaction with ARNT.

GST-pull-down and in vivo co-immunoprecipitation studies have demonstrated a direct interaction between AHR and ER (2,41) as well as ARNT and ER (1). Furthermore, dominant negative studies have shown that the AHR TAD and the N-terminal region of ER can reciprocally repress transcription from ERE- and DRE-driven luciferase constructs, respectively (42). TCDD exposure causes a robust increase in mRNA levels and of DRE-driven reporter gene activity, both of which are significantly decreased by co-treatment with E2 in Hepa-1 and MCF-7 cells (43). Likewise, in the human endometrial carcinoma cell line ECC-1, E2 again blocked DRE-driven luciferase activity as well as 7-ethoxycoumarin hydroxylation (44). This effect was reversed by the addition of the selective ER modulator (SERM). 4-hydroxy-tamoxifen, (TAM). Despite a wealth of evidence suggesting that ER activation represses AHR target genes, little is known regarding the molecular mechanisms involved. Furthermore, most experimental efforts to characterize the biochemical pathways involved in ER-AHR cross-talk have focused on the TCDD-inducible repression of ER target genes.

**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 (42). We have confirmed and extended these observations in HEK-293 cells and in an *in vitro* system. However, these systems cannot distinguish between direct transcriptional events at the target and secondary transcriptional events such as the transcriptional activation/repression of other regulatory genes. Therefore, 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 100 nM E2 caused a significant increase in *Cyp1a1* gene transcription in MCF-7 cells (Figure 2a). 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 2b). The addition of 100 nM estradiol significantly reduced TCDD induced *Cyp1a1* gene transcription in the presence or absence of cycloheximide (Figure 2a 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. Initially, consistent with other investigators’ observations, we observed the presence of AHR and ARNT over the human *Cyp1a1* enhancer in a TCDD-dependent fashion (1,2,23). ERα was greatly enriched at the *Cyp1a1* enhancer only after treatment with a combination of 1 nM TCDD and 100 nM E2 (Figure 3b). As a control, we monitored the presence of AHR, ARNT and ERα over the *pS2* promoter under similar conditions (Figure 3b) with results similar to those observed by other investigators (2). 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 3b), 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* expression observed in MCF-7 cells (data not shown). Affinity purified antibody to the hemaglutinin protein tag (control antibody) failed to precipitate either the *Cyp1a1* enhancer or the *pS2* promoter, indicating that anti-AHR, -ARNT and –ERα were precipitating their respective antigens in a specific fashion. Taken together, these data indicate that liganded ERα associates with the *Cyp1a1* enhancer only after TCDD activated transcription has been initiated.

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. Chromatin samples from cells treated with either, vehicle, E2, TCDD, or E2+TCDD were precipitated with either anti-AHR or anti-ERα. 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 3a). 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. This is in direct contrast to the observations of another group that failed to record ERα on the *Cyp1a1* enhancer in response to co-treatment with 3-methylcholanthrene (3-MC), a PAH and E2 (2). We cannot resolve these differences except to note that in the study noted above the AHRC was activated with the PAH, 3-MC and that ligand-specific differences may exist.

The nature of repression by tethering of ligand activated NR’s is not well understood. The classical model of NR
activation by ligand suggests that ligand facilitates an exchange of NCoR/SMRT/Sin2/HDAC co-repressor complexes for co-activator complexes with ATP-dependent chromatin remodeling and histone acetyl-transferase activities (46,47). It has been suggested that this happens only in the context of a receptor’s own cognate positive response element. Studies regarding the repression of thyrotropin β gene by TR suggest that this switch does not occur in the context of a negative TRE (48). Other studies have suggested that GR-mediated repression of the IL-8 gene occurs through a direct protein-protein interaction with the NF-κB heterodimeric transcription factor, and is independent of GRE binding (49). One model put forth is that GR recruits GRIP1 in this context, but a steric change in the complex due to tethering unmasks GRIP1’s repressor function (50). This, in turn, may play a role in the GR-mediated inhibition of phosphorylation of the carboxy-terminal catalytic domain of RNA polymerase II (51). Whether ERα utilizes GRIP or recruits other ancillary factors remains unclear, however, our data would suggest that ultimately, histone acetylation and chromatin condensation occurs.

The mechanism by which PAH mediate adverse biological effects, is understood in broad terms. PAH induce expression of Cyp1a1 and Cyp1b1 via the AHRC. These cytochrome P450 xenobiotic-metabolizing enzymes generate electrophilic derivatives that form DNA adducts thereby activating proto-oncogenes and inactivating tumor suppressor genes. However, P450 induction is also responsible for the ultimate metabolism and clearance of many toxic substances. Furthermore, repression of PAH and HAH inducible gene transcription by activated ERα could, in susceptible tissues lead to a blunted or inappropriate response to these carcinogens, such as the unmasking of other drug metabolizing activities leading to an increased or untoward production of electrophilic metabolites. Conversely, breast cancer treatments that include ER antagonists, such as tamoxifen might increase or exacerbate AHR activity in tumor cells. Furthermore, AHR is important for development. Exposure to estrogens in an untoward fashion (i.e. pseudo-, and phyto-estrogens), or altered ER status in affected tissues (i.e. certain small cell lung tumors, breast cancer) would ultimately impact AHR function and disrupt physiological homeostasis.

We have demonstrated that ERα can repress AHRC target gene induction through direct protein-protein interactions with the AHR. Endogenous or exogenous ligand availability for both AHR and ER as well as target tissue and receptor availability will likely determine the degree of ERα transrepression. Furthermore, the identification of ancillary factors recruited during transrepression of AHRC-mediated gene induction highlights an intriguing avenue for future research.

Footnotes

¶ This work was supported by NIEHS, National Institutes of Health Grant ES04869.

1 The abbreviations used are: AHR, aryl hydrocarbon receptor; AHRC, aryl hydrocarbon receptor complex (AHR/ARNT); ARNT, aryl hydrocarbon receptor nuclear translocator; CBP, CREB-binding protein; p/CIP, p300/CBP-interacting protein; ChIP, chromatin immunoprecipitation; DBD, DNA-binding domain; ERα, estrogen receptor-alpha; GRIP1, glucocorticoid receptor-interacting protein; HSP90, heat-shock protein 90; kDa, kilodalton; NCoA-2, nuclear co-activator-2; NR, nuclear receptor; RIP140, receptor interacting protein140; SRC-1, steroid receptor co-activator-1; TCDD, 2,3,7,8-tetrachlorodibenzop-dioxin; TR, thyroid hormone receptor; TRIP230, thyroid hormone receptor/retinoblastoma protein-interacting protein; UAS, upstream activating sequence; XAP2, hepatitis B virus X-associated protein 2, XRE, xenobiotic response element.
Acknowledgments: We would like to thank Dr. Oliver Hankinson for the rabbit anti-ARNT antibody.

Figure Legends

Figure 1. ERα interactions with AHR and ARNT. (A) In vitro translated ERα interacts with GST-ARNT and GST-AHR-P/S/T fusions. (B) Effect of ERα on wild-type AHR and AHRΔP/S/T mediated transcription. HEK-293 cells were maintained in serum- and Phenol Red-free media for at least 48 h prior to any experimental manipulation. (C) ERα represses ARNT transactivation function and enhances AHR transactivation. A schematic of AHR/ARNT-GAL4-DBD mutants used in mammalian interaction studies is presented above. GAL4 DNA-binding domain (GAL4DBD), the basic-Helix-Loop-Helix (bHLH), PAS A and B (A,B), acidic (Ac), Q-rich (Q), and Proline-Serine-Threonine-rich (P/S/T) regions are shown. (D) HEK-293 cells were co-transfected with expression cDNA plasmids encoding either GAL-AHR419-805 or GAL-ARNT and ERα and the GAL4 driven luciferase vector, pG5E4T (100 ng each). After transfection cells were treated with 100 nM E2 to activate ERα. Cells were grown for an additional 18-20 h, harvested and luciferase activity was determined. Luciferase activity was normalized to that of β-gal to control for transfection efficiency.

Figure 2. 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 cycloheximide (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 Me2SO (C), 100 nM 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.

Figure 3. 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).

Figure 1

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