Distinct Roles for Aryl Hydrocarbon Receptor Nuclear Translocator and Ah Receptor in Estrogen-Mediated Signaling in Human Cancer Cell Lines

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Abstract
The activated AHR/ARNT complex (AHRC) regulates the expression of target genes upon exposure to environmental contaminants such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Importantly, evidence has shown that TCDD represses estrogen receptor (ER) target gene activation through the AHRC. Our data indicates that AHR and ARNT act independently from each other at non-dioxin response element sites. Therefore, we sought to determine the specific functions of AHR and ARNT in estrogen-dependent signaling in human MCF7 breast cancer and human ECC-1 endometrial carcinoma cells. Knockdown of AHR with siRNA abrogates dioxin-inducible repression of estrogen-dependent gene transcription. Intriguingly, knockdown of ARNT does not affect TCDD-mediated repression of estrogen-regulated transcription, suggesting that AHR represses ER function independently of ARNT. This theory is supported by the ability of the selective AHR modulator 3',4'-dihydroxy-α-naphthoflavone (DINM) to repress estrogen-inducible transcription. Furthermore, basal and estrogen-activated transcription of the genes encoding cathepsin-D and p52 are down-regulated in MCF7 cells but up-regulated in ECC-1 cells in response to loss of ARNT. These responses are mirrored at the protein level with cathepsin-D. Furthermore, knock-down of ARNT led to opposite but corresponding changes in estrogen-stimulated proliferation in both MCF7 and ECC-1 cells. We have obtained experimental evidence demonstrating a dioxin-dependent repressor function for AHR and a dioxin-independent co-activator/co-repressor function for ARNT in estrogen signalling. These results provide us with further insight into the mechanisms of transcription factor crosstalk and putative therapeutic targets in estrogen-positive cancers.

Unliganded AHR exists in the cytoplasm as part of a multimeric complex containing two molecules of HSP90, the HSP90 co-chaperone p23, and hepatitis B virus X-associated protein 2 (XAP2) [7,8,9,10]. 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 regulatory proteins, such as steroid receptor coactivator-1 (SRC-1), CREB binding protein (CBP/p300), NCoA2/GRIP1 [11,12], receptor-interacting-protein 140 (RIP140) [13], CoCoA [14], GAC63 [15], NcoA4 [16] and TRIP230 [17], which play significant roles in determining the activity of TCDD-induced gene transcription. These co-activators and co-repressors incorporate themselves into multimeric complexes that modify chromatin structure, stabilize core transcriptional machinery, and mediate RNA chain elongation [18]. In addition to these classic transcriptional co-activators and co-repressors, AHR is recruited by other transcription factors during transcription, including estrogen receptor-α (ERα) [11,19,20] and NF-κB [21] to modify their intrinsic activities.

Introduction
Elucidating the mechanisms underlying transcription is crucial to our understanding of how cells and organisms respond to physiological signals and environmental stimuli. The aryl hydrocarbon receptor (AHR) and the aryl hydrocarbon receptor nuclear translocator (ARNT) are members of the basic helix-loop-helix/PER-ARNT-SIM (bHLH-PAS) family of proteins and form a heterodimeric transcription factor upon binding a variety of environmental contaminants, including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) [1]. The activated AHR/ARNT complex (AHRC) plays key roles in carcinogenesis and regulates the expression of Cytochrome P450IA1 (CYP1A1) and other xenobiotic target genes to combat the effects of environmental contaminants [1]. In addition, AHR has been shown to be important for normal development and physiological homeostasis [2,3,4] and is essential for certain functions of the immune response, such as regulation of interleukin-17 producing T-helper cells [5] and induction of the cytokine interleukin-6 in MCF7 breast cancer cells [6].
ERα/β are ligand-activated transcription factors that belong to the superfamily of nuclear hormone receptors (NR) [22] and bind 17β-estradiol (E2) to regulate genes involved in reproduction and cellular growth and proliferation [23]. Upon ligand binding, ER forms a functional homodimer and binds its cognate response elements. Interestingly, there is a ligand-dependent reciprocal disruption between ER and AHR signaling. For instance, activated ERs inhibits AHR activity at CYP1A1 through direct protein-protein interactions, termed transrepression [11]. Conversely, TCDD’s anti-estrogenic properties are well documented as it represses the E2-inducible genes pS2 and cathepsin-D (CAT-D) [24,25,26]. However, the mechanisms of repression occurring at E2-responsive genes are unclear. Proposed theories for this repression include: (i) competition for a common pool of co-activators [27]; (ii) a direct down-regulation of CAT-D transcription through upstream inhibitory dioxin response elements [28]; (iii) activation of a TCDD-inducible inhibitory factor [28]; (iv) an AHR-dependent E3-ligase that degrades proteins crucial for ER-signalling [29], or; (v) a direct transrepression interaction between AHR and ER [11,19].

In this study, we examined the role of AHR and ARNT on ERα-dependent target gene transcription in the human MCF7 breast cancer and the human ECC-1 endometrial-cervical cancer cell lines. Our data suggest that AHR and ARNT act independently from each other at off-target sites and we revealed that ARNT is not essential for TCDD-dependent repression of ER-signaling. In addition, we have demonstrated that ARNT acts as a cell specific coactivator in MCF7 cells, and as a corepressor in ECC-1 cells. Finally, we show that ARNT knockdown not only affects accumulation of mRNA and protein of ERα-target genes, but also has phenotypic consequences by influencing ERα-mediated cell proliferation.

Results

AHR-dependent repression of estrogen signaling in ECC-1 cells

The presence of AHR, ARNT and ERα at the dioxin-inducible CYP1A1 enhancer and the E2-inducible pS2 promoter has already been documented in MCF7 cells and other breast cancer cell lines [11,19,20,30]. Although preliminary studies have identified ECC-1 human endometrial cells as an ideal system to study dioxin signaling through upstream inhibitory dioxin response elements [28], studies of CYP1A1 enhancer activity are scarce. AHR has been shown to repress pS2 expression in ECC-1 cells (Figure 1A). Furthermore, the recruitment of ERα to the pS2 promoter occurs in an E2-dependent fashion while AHR and ARNT are present after treatment with either ligand but are enriched during co-treatment (Figure 1B).

We have previously shown that ERs associates with the AHRCC to mediate estradiol-dependent transrepression of dioxin-inducible gene transcription [11]. Therefore, we set out to determine the functional significance of AHR in estrogen-inducible gene transcription. We transfected ECC-1 cells with small inhibitory RNA directed towards either AHR (siAHR) or a GFP negative control (siGFP). After transfection, cells were serum starved for 24 h, followed by 24 h ligand treatment and then harvested for total mRNA that was quantified through quantitative PCR. As expected, ablation of AHR and co-treatment with 2 nM TCDD and 10 nM E2 results in the loss of TCDD-induced repression of pS2 transcription (Figure 1C). However, loss of AHR had no measurable effect on basal, or estrogen activated pS2 mRNA accumulation. As a control for siRNA specificity, Western blots of AHR protein show a greater reduced expression of AHR protein after transfection and unchanged protein levels of ERα and XAP2 which served as a loading control (Figure 1E). Thus, these demonstrate the specificity of the siRNAs to AHR, and that loss of AHR does not affect accumulation or turnover of basal ERα protein levels. Furthermore, the induction of CYP1A1 transcription with TCDD after AHR knockdown is attenuated when compared to the siGFP negative control (Figure 1D). Thus, AHR is a requirement for TCDD-induced repression of E2-responsive gene transcription and the increased transcriptional response with combinatorial treatment is due solely to the loss of AHR and other putative transcriptional modifiers associated with its presence. Finally, we obtained essentially identical results in both MCF7 and ECC-1 cell lines under serum-starved or charcoal-stripped serum conditions suggesting that differences in the cell lines’ ability to go through cell cycle does not impact this phenomenon. Together, these results support the concept that the ECC-1 cell line is an excellent alternative cell model to study dioxin-induced disruption of estrogen receptor signaling.

ARNT has cell specific co-activator/co-repressor functions

The identification of ARNT as a co-activator in estrogen signalling [27,30], juxtaposed with the known transrepressor effects of TCDD, led us to investigate the role played by ARNT in TCDD-mediated transrepression of ER function in various human cancer cell lines, namely MCF7 and ECC-1 cells. Through siRNA directed towards ARNT and a scrambled negative control (siSCX), and subsequent qPCR analysis of endogenous pS2 and CAT-D gene transcription, we made several interesting observations. First, ARNT displays co-repressor properties in ECC-1 cells. Accumulation of pS2 (Figure 2A) and CAT-D (Figure 2B) mRNA levels are exacerbated during E2 treatments after loss of ARNT suggesting a cell specific function for ARNT independent of AHR. Furthermore, we observed this phenomena with three separate siRNAs directed towards ARNT (siRNA’s 1 and 3 are shown in Figure 2A, B and C). We used at least two siRNA’s for all other experimental parameters with essentially identical results, but for brevity, hereafter we only present data depicting the use of one siRNA. Secondly, consistent with the findings of several other investigators, ARNT displayed co-activator properties in MCF7 cells [27,30]. Indeed, knockdown of ARNT protein dampens E2-induced transcription of pS2 (Figure 2C) and CAT-D (Figure 2D). Finally, in a dose response experiment, we observed a concomitant decrease of CAT-D protein levels with increasing concentrations of TCDD in both ECC-1 and MCF7 cells (Figure 2E). These data indicate that ARNT function as it relates to ER signaling is likely dictated by other factors specific to the cellular environment.

To determine if the modulation of transcriptional activity translated into similar protein expression profiles, we used Western blot analysis to visualize the level of CAT-D protein after ARNT knockdown. Cells were transfected and ligand treated under identical conditions as the qPCR parameters. When compared to
the scrambled negative control, CAT-D protein expression after E2 treatment is exacerbated in ECC-1 cells with ARNT knockdown (Figure 3A). Conversely, MCF7 cells transfected with ARNT siRNA resulted in a blunted expression of CAT-D protein during E2 treatment (Figure 3C). Furthermore, ERα levels remained consistent, regardless of ARNT status (Figure 3A and C). Most importantly, dioxin-induced repression of ER signaling was maintained at the protein level after ARNT knockdown (Figure 3A and C). The representative blots were normalized to α-tubulin and luminescence was measured using GeneTools 4.01.2 software (Syngene). These normalized values showed a two-fold induction of CAT-D in ECC-1 cells with ARNT knockdown after E2 treatment (Figure 3B, columns 2 and 6), whereas in MCF7 cells, knock-down of ARNT resulted in a 40% decrease in E2-dependent CAT-D expression (Figure 3D, columns 2 and 6). These results provide concrete evidence that the level of protein
Figure 2. Loss of ARNT shows a cell specific co-activator or co-repressor transcriptional function. ECC-1 cells (A and B) and MCF7 cells (C and D) were transfected with either scrambled siRNA (siSCX) or siRNA directed to ARNT (siARNT#1 or siARNT#3). Twenty-four hours after transfection, cells were treated with vehicle (DMSO), E2 (10 nM), TCDD (2 nM) or a combination of E2 and TCDD. Gene expression was determined by
expression mirrors the transcriptional response in both cell lines and that physiological consequences must ensue with loss of ARNT.

These surprising results were coupled with the observation that TCDD-induced repression of ER-signaling is maintained in both cell lines after ARNT knockdown (Figure 2 and 3) thus providing evidence that ARNT is not required for TCDD/AHR-dependent repression of ERα signaling. This hypothesis is supported by the ability of a selective aryl hydrocarbon receptor modulator (SAHRM) to repress estrogen signaling. We examined the ability of the known SAHRM, DiMNF [33,34] to repress E2-mediated transcription by RT-PCR in MCF7 and ECC-1 cells (Figure 4). DiMNF was as effective at repressing CAT-D mRNA accumulation as TCDD. DiMNF is a potent antagonist of AHR and

Figure 3. Effect of ARNT knockdown on ARNT, CAT-D and ERα protein levels. ECC-1 (A and B) and MCF7 (C and D) cells were transfected with either siSCX or siARNT and ligand treated as described in Figure 2. (A and C) Representative Western blots of ARNT, CAT-D, ERα and α-tubulin protein levels. Bar graphs of CAT-D protein levels in ECC-1 (B) and MCF7 (D) cells after normalizing luminescence values to α-tubulin. Open bars represent ligand treatments after siRNA to scrambled negative control (siSCX) and closed (black) bars represent ligand treatments after siRNA to ARNT (siARNT). Experiments were performed three times with essentially identical outcomes.

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ARNT-independent. thus, it seems likely that AHR’s transrepression function is entirely ARNT-independent.

ARNT knockdown causes increased proliferation in ECC-1 cells and decreased proliferation in MCF7 cells

Sensitivity to estrogen has been linked to proliferation and cell transformation in ER-positive carcinoma cells [35]. To establish if ARNT can influence E2-dependent cellular proliferation, we performed proliferation assays on ECC-1 and MCF7 cells after ARNT siRNA treatment. Consistent with our quantitative PCR and Western blot data, ECC1 and MCF7 cells displayed altered rates of proliferation after ARNT knockdown compared to cells transfected with the scrambled negative control siRNA (Figure 5A and B). Knockdown of ARNT was monitored for 72 h and the significant knock-down observed was unchanged essentially at each time point (Figure 5C). Neither cell line displayed altered growth patterns until the 48 hour time point. At 48 hours, ECC-1 cells in both the siSCX and siARNT conditions showed modest proliferation in response to E2 treatments. At the 96 hour time point, there was a highly significant increase in E2-inducible proliferation of ECC-1 cells treated with siARNT, compared to scrambled control treated cells. Intriguingly, ARNT knockdown increased basal growth rates and caused an exacerbated response to E2 with the cell number nearly doubling in the siARNT E2 treatments compared to the siSCX E2 treatments. Conversely, MCF7 cells showed a decreased proliferation rate after ARNT knockdown (Figure 5B). The growth rates were not significantly different until the 48 h time point, when the E2-inducible proliferative response in the scrambled negative control was apparent. The siARNT transfected cells had a blunted growth response during both control and E2 conditions. At 96 hours, the cells began losing sensitivity to E2 and entered into a senescent state. Whether or not this was due to the growth conditions is unclear. However, these data further support the hypothesis that ARNT has co-repressor properties in ECC-1 cells and co-activator properties in MCF7 cells. Moreover, the sensitivity to E2 and the reciprocal growth responses exhibited by the two cell lines are bona fide phenotypic consequences of ARNT ablation.

Discussion

Many environmental contaminants that serve as activators of the aryl hydrocarbon receptor are known as putative endocrine disrupting compounds. Exposure to many of these compounds occur on a daily basis and represents significant risks to human health. In particular, the repressive effects elicited by ligands of the AHR on ER signaling have been well documented [11,19, 36,37,38]. Other reports have described the co-activator potential of ARNT for ER-mediated transcription [27]. Despite the growing body of evidence to support roles for AHR and ARNT in ER function, little is known about the normal physiological role of these proteins as they relate to estrogen signaling or the molecular determinants of toxicant-induced transrepression of ER function by AHR. Finally, this investigation revealed that ARNT is not essential for AHR-mediated off-target transrepression. In order to delineate the molecular mechanisms underlying AHR-mediated transrepression we sought to uncouple AHR and ARNT function in ER-positive human cancer cell lines. In doing so, we discovered that ARNT attenuates activated ER-target gene transcription in ECC-1 cells, in direct contrast to its co-activator function described in MCF7 cells [27].

ARNT was originally identified as a bona fide transcription factor [39] and dimerization partner of AHR [40]. Beyond its canonical transcription factor function, ARNT can interact with several other transcription factors, including ERα [11] and its co-activator function for ER signaling has been well described [27,30]. Therefore, we expected that knockdown of ARNT in ECC-1 endometrial-cervical cancer cells would result in a diminution of ER target gene expression. The resulting increase in mRNA accumulation, protein expression and E2-inducible proliferation strongly suggests that ARNT acts as a transcriptional co-repressor in this cell line. The molecular mechanism(s) underlying the differences observed in MCF7 and ECC-1 cells likely is related to differences in the nature and composition of the ancillary transcriptional machinery recruited by ARNT in each cell line. ARNT presents several different protein-protein interactions and combinations that cannot be assessed by a simple in silico search for known protein-protein interactions.
interaction domains for the recruitment of co-activator proteins, including a carboxy-terminal transactivation domain [18], its PAS-B region [41] and its basic-helix-loop-helix domain [12]. In addition, an association between ARNT and the transcriptional co-repressor protein, SMRT has been demonstrated [42]. However, we believe that this is the first demonstration that ARNT has dual co-activator/co-repressor functions in a cell-specific fashion (Figure 6). In addition, the repercussions of these transcriptional effects can be observed at the translational and phenotypic levels.

Several models have been hypothesized to explain the molecular mechanisms underlying transcription factor mediated cross-talk, particularly the ability of one transcription factor to repress another’s function [18]. Reugg and colleagues, among others, have suggested that transcription factor-mediated transrepression might be the result of competition for a limited pool of co-activator proteins [27]. However, the identification of ARNT as a co-repressor in ECC-1 cells unmasks a more complex mechanism of transrepression at E2-inducible genes than the co-activator competition model suggests. While these data do not definitively disprove this model, we believe that our data suggest that it is unlikely because co-activator competition cannot account for TCDD-inducible repression in ECC-1 cells as activated AHR should squelch ARNT’s co-repressor function. In addition, ARNT, a protein that has demonstrated the ability to recruit numerous co-activators [12,15,17,41,43,44] should illicit an effect similar to ligand-activated AHR if co-activator pools were in such limited supply that competition would hinder individual transcription factor function. This clearly is not the case in the ECC-1 cell line.

The experimental evidence presented above indicates that AHR does not require ARNT to mediate its off-target transrepressor...
effects. Loss of ARNT in both MCF7 and ECC-1 cells failed to abrogate the repressive effects of TCDD on E2-inducible transcription and protein expression (Figures 2 and 3). The effects DiMNF-bound AHR are independent of dioxin response element binding as nuclear extract from cells treated with DiMNF does not retard the movement of a labeled dioxin response element probe in gel shift assays [34]. Furthermore, AHR-ARNT dimerization is a requirement for DNA binding, suggesting that this may not occur in the presence of DiMNF. This represents a paradigm shift in our understanding of AHR function and has implications for the use and effectiveness of selective AHR modulators. Indeed, a novel AHR antagonist has proven to be a potent stimulator of AHR-dependent repressor of cytokine signaling [33,34]. Furthermore, DiMNF was effective in repressing ER-regulated target gene expression (Figure 4). Molecular modelling studies demonstrate that DiMNF forms an extra hydrogen bond with AHR at Thr289 [34], a characteristic not shared with the partial agonist α-naphthoflavone suggesting that the DiMNF-AHR adopts a unique confirmation. Thus, the flavonoids represent a class of compounds that may be attractive targets for further testing and development to determine their effects on ER target gene expression.

Our experimental evidence demonstrates a TCDD-dependent repressor function for AHR and a TCDD/AHR-independent co-activator/co-repressor function for ARNT in estrogen signaling. These results provide us with further insight into the mechanisms of transcription factor crosstalk and putative therapeutic targets in estrogen-positive cancers. Taken together, our data suggest a more complex mechanism of ARNT function and AHR-mediated

Figure 6. A schematic representation of estrogen signaling, describing the transcriptional functions of ER, AHR and ARNT in ECC-1 and MCF7 cells. The presence of E2 facilitates the assembly of transcriptional modifiers that induce transcription of E2-responsive genes. Ligand activated AHR represses ER-signaling independent of ARNT. The loss of ARNT in ECC-1 cells, where it acts as a co-repressor, leads to increased sensitivity to E2 and increased transcriptional activity at ER regulated genes. The loss of ARNT in MCF7 cells, where it acts as a co-activator, leads to decreased sensitivity to E2 and decreases transcriptional activity at ER regulated genes.

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transrepression of ER-signaling than previously suggested. The clinical utility of these findings remains to be tested and will be the subject of future investigations.

Materials and Methods

Materials and Cell culture

3′,4′-dimethoxy-α-naphthoflavone (DiMNF) was obtained commercially [Indofine Chemical Co., Hillsborough, NJ]. ECC-1 and MCF7 cells (ATCC) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, BioWhittaker, Lonza) with 10% fetal bovine serum (FBS, HyClone, PerBio, Thermo Fisher Scientific Inc.) and supplemented with 100 units/ml potassium penicillin-100 μg/ml streptomycin sulphate (BioWhittaker, Lonza) at 37°C, 20% O2, and 5% CO2. Twenty-four h before any experimental perturbation, cells were washed 2× with PBS, and maintained in Phenol-Red-free media without FBS [46].

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation (ChIP) assays were performed as described previously [17]. Briefly, cells were plated into 150 cm2 dishes immunoprecipitation (ChIP) assays were performed as described previously. Cells were washed 2 times with PBS, trypsinized and seeded into 12-well plates at 10,000 cells/well in DMEM with 2.5% charcoal-stripped FBS. Twenty-four hours after plating, E2 was added directly to half the wells to a final concentration of 10 nM. Cells were counted three times. Determinations were performed in triplicate and each sample was counted three times.

Western blot analysis

In order to determine the effects of TCDD on E2-inducible protein levels, MCF7 and ECC-1 cells were treated either with vehicle (Me2SO), TCDD (2 nM), E2 (10 nM), or a combination of TCDD and E2 for 24 h. For protein analysis that included siRNA treatment, cells were transfected for 6 h and then starved in seric free media for 24 h prior to ligand treatments. Cells were harvested and the protein concentration determined by the RC DC protein assay (Bio-Rad). Equal amounts of proteins from the samples were resolved on a SDS-acrylamide gel then transferred to polyvinylidene fluoride (PVDF) membrane. Upon completion of transfer, the membrane was wetted with 100% methanol then probed with anti-ARNT (goat polyclonal IgG; Santa Cruz Biotechnology, Inc.), anti-AHR (rabbit polyclonal, Biomol, GmbH), anti-CAT-D (rabbit polyclonal IgG; Santa Cruz Biotechnology, Inc.), anti-ERα (rabbit polyclonal IgG; Santa Cruz Biotechnology, Inc.), anti-α-tubulin (mouse monoclonal IgG; Santa Cruz Biotechnology, Inc.) or anti-XAP2 mouse monoclonal antibody. The detection was performed using horseradish peroxidase conjugated anti-mouse or anti-rabbit or anti-goat IgG and ECL detection kit (GE Healthcare).

Proliferation Assay

MCF7 cells and ECC-1 cells at 75–80% confluency were transfected with either ARNT or scrambled negative control siRNA. After 6 h, cells were washed 2 times with PBS, trypsinized and seeded into 12-well plates at 10,000 cells/well in DMEM with 2.5% charcoal-stripped FBS. Twenty-four hours after plating, E2 was added directly to half the wells to a final concentration of 10 nM. Cells were counted at 0 (control), 12, 24, 48 and 96 h following E2 administration. A second treatment of E2 (10 nM) was added to the cells at 48 hrs. Determinations were performed in triplicate and each sample was counted three times.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 4.0. For multiple comparisons (i.e. siRNA experiments) statistical significance was determined using a 2-way ANOVA with Tukey’s Multiple Comparison test. Values are presented as means ± standard error of the mean (SEM). A P value<0.05 was considered to be significant.

Author Contributions

Conceived and designed the experiments: TVB GHP GGP. Performed the experiments: MPL MKT BDH. Analyzed the data: TVB MPL GGP. Contributed reagents/materials/analysis tools: TVB GHP GGP. Wrote the paper: MPL TVB GHP GGP BDH.
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