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Four and a half LIM domain 2 alters the impact of aryl hydrocarbon receptor on androgen receptor transcriptional activity

Alexandra Kollara [a,b], Theodore J. Brown [a,b,*]

[a] Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada M5T 3H7
[b] Division of Reproductive Endocrinology and Infertility, Department of Obstetrics and Gynecology, University of Toronto, Toronto, Ontario, Canada M5S 3G5

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ABSTRACT

Aryl hydrocarbon receptor (AhR) ligands modulate androgen receptor (AR) signaling in prostate cancer cells through partially defined mechanisms. Furthermore, these facilitatory and inhibitory effects of AhR on AR signaling appear to be cell or context specific. In the present study we demonstrate that both AhR and AhR-nuclear translocator (ARNT) interact with AR. AhR but not ARNT enhanced the AR-transcriptional activity which was independent of exogenous AhR ligand treatment (2,3,7,8-tetrachlorodibenzo-*p*-dioxin, TCDD). We then tested if coactivators common to both receptors alter the facilitatory effect of AhR on AR activity. NcoA4 overexpression did not alter the AhR facilitatory effect on AR, whereas SRC1 overexpression further enhanced the effect. In contrast, FHL2 overexpression blocked the facilitatory effect of AhR. In the presence of exogenous FHL2 expression, AhR repressed AR activity, whereas at low endogenous levels of FHL2 expression, AhR overexpression enhanced AR activity. At high FHL2 expression levels, TCDD treatment decreased AR activity and this effect was reversed by AhR overexpression. These findings demonstrate that AhR modulation of AR activity is differentially altered by the level of FHL2 and AhR present in the cell.

1. Introduction

The aryl hydrocarbon receptor (AhR) mediates the transcriptional effects of several environmental toxins including recognized carcinogenic agents. In addition to altering transcription of direct target genes such as cytochrome P450 enzymes, AhR interacts with estrogen (ER) and androgen (AR) receptors to modulate their ability to regulate gene transcription [1–3]. These interactions have implications on our understanding of how environmental toxins influence hormone-dependent cancers, particularly that of the breast and prostate.

An increased rate of prostate carcinogenesis in the TRAMP (transgenic adenocarcinoma of the mouse prostate) mouse model in the absence of AhR expression was recently demonstrated [4], suggesting a protective role of AhR in prostate cancer. This is supported by studies showing that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), a potent AhR agonist, inhibits the growth of human LNCaP prostate cancer cells [2,5,6] and blocks androgen-induced prostate specific antigen (PSA) expression in these cells [2,3,6]. In addition to TCDD, other environmental contaminants such as benzo[a]pyrene (BaP) and 7,12-dimethylbenz[a]anthracene (DMBA) have also been shown to exert antiandrogenic effects [2,3,7].

The AhR has a dual effect on AR activity, exhibiting both facilitatory and inhibitory effects that appear to be cell or context specific. Studies by Ohtake et al. [8] indicate that constitutively active AhR enhances AR-transcriptional activity in 293T kidney cells, perhaps functioning as an AR coactivator, but inhibits dihydrotestosterone (DHT)-induced AR activity in LNCaP cells [9]. Morrow et al. [6] have reported a similar effect in LNCaP cells but found no effect of AhR ligands on AR activity in ZR-75 breast cancer cells. Recent evidence has suggested that ligand-activated AhR acts as an adaptor component for cullin 4B, targeting steroid hormone receptors for ubiquitination and proteosomal degradation [9]. Decreased androgen sensitivity through increased degradation of AR protein provides an attractive model whereby androgen responses are inhibited by AhR ligands. Additionally, ligand-activated AhR inhibition of androgen-induced proliferation of LNCaP cells [2,5,6] has been associated with decreased expression of cyclin D1 and p21 [5], leading to inhibition of androgen-mediated phosphorylation of retinoblastoma protein and decreased cell growth.

Additional mechanisms similar to those proposed to explain the inhibitory effects of TCDD on ER activity [10,11], including competition for coregulatory proteins [12,13], could play a role in modulating AR signaling. We have recently shown that enhanced AhR signaling by nuclear receptor coactivator 4 (NcoA4) or four

* Corresponding author at: Samuel Lunenfeld Research Institute, Mount Sinai Hospital, 25 Orde Street, Box 41, Toronto, Ontario, Canada M5T 3H7.
Tel.: +1 416 586 4800x2696; fax: +1 416 586 5130.
E-mail address: brown@lunenfeld.ca (T.J. Brown).

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and a half LIM domain 2 (FHL2) is diminished in the presence of AR [14,15]. Since these coactivators also regulate the activity of AR [16,17], these findings raised the possibility that competition for coactivators may similarly influence AR signaling.

In this study, we examined the role of AhR–AR interaction and the impact of FHL2, NcoA4, and SRC1 on AhR mediated effects on AR-transactivational activity. For many of these experiments, we used PC-3 cells transfected with a full-length wild-type AR cDNA to avoid potential confounding effects of AR mutations such as expressed by LNCaP cells, which result in diminished steroid specificity [18]. In addition, parental PC-3 cells contain higher endogenous levels of AhR and ARNT [14] compared to LNCaP cells; thus, they are a well-suited model to examine the impact of AhR/ARNT interaction on AR signaling. Our results indicate that AhR facilitates or inhibits AR-transactivational activity in a manner that is determined by levels of FHL2 or AhR expression in the cell.

2. Material and methods

2.1. Cell lines and reagents

COS cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco-BRL, Gaithersberg, MD). PC-3 parental and PC-3(AR) cells were grown in RPMI 1640 medium without phenol red supplemented with 5% heat-inactivated charcoal-stripped FBS [19]. All media were supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin and 0.625 μg amphotericin B/ml (all from Gibco-BRL). 5α-Androstan-17β-ol-3-one (dihydrotestosterone, DHT) obtained from Sigma Chemical Co. (St. Louis, MO) was dissolved in ethanol. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin dissolved in 100% n-nonane was obtained from Cambridge Isotope Laboratories Inc. (Andover, MA) and was diluted in ethanol. Both DHT and TCDD were further diluted with culture medium before addition to cell cultures.

2.2. Co-immunoprecipitation studies

Cells were harvested using 1% trypsin and resuspended in 1.0 ml of ice-cold lysis buffer (50 mM Tris, pH 8.0, 120 mM NaCl, 0.5% (v/v) NP-40, containing complete protease inhibitor cocktail; Roche, Laval, QC). The lysates were then centrifuged at 20,000 × *g* for 15 min at 4 °C and the resulting supernatant (3 mg/ml total protein) was precleared with protein A-Sepharose 4B (Invitrogen, Burlington, ON) and then immunoprecipitated using 1 μg/ml of AhR rabbit polyclonal antibody (Biomol Research Laboratories, PA) or ARNT rabbit polyclonal antibody (Novus Biologicals Inc., Littleton, CO) or normal rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, California; used as a negative control) as previously described [14]. The membranes were probed with anti-AR (1:200; Novocastra Laboratories, Burlington, ON) antibody. Immunoreactive band intensities were quantified using Image Quant version 5.0 software.

2.3. Reporter gene studies

Expression vectors containing full-length human AR (pcDNA3-AR), NcoA4 (pSG5-NcoA4), or FHL2 (pcDNA3/Hygro(+)-FHL2) cDNA were generated as previously described [14,19]. Expression vectors for SRC1 (pCR3.1-hSRC-1) and AhR (pcDNA3-AhR) were kindly provided by Dr. B.W. O'Malley (Baylor College of Medicine) and Dr. O. Hankinson (University of California), respectively. An expression vector containing full-length ARNT cDNA (pcDNA3-ARNT) was generated by ligating a 2574 bp BamHI/HindIII digest from pcDNAI/neo-ARNT (provided by Dr. O. Hankinson) into the corresponding sites of pcDNA3.

COS, PC-3 or PC-3(AR) cells were seeded into 24-well plates at a density of 5×10^4 cells/well and the cells were transiently transfected with a luciferase reporter construct driven by the PSA promoter (pGLPSAp5.8; provided by Dr. A. Mizokami, Kanazawa University, Japan [20]) along with expression constructs for AR, AhR, ARNT, SRC1, NcoA4 and/or FHL2 as indicated for each experiment. Transfections were performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's recommended protocol. The total amount of total plasmid DNA used was normalized to 0.9 μg/well by addition of empty vector. The transfected cells were treated with 1 or 10 nM DHT and/or 10 nM TCDD or vehicle 24 h after transfection and harvested 24 h later. The cell lysates were prepared using reporter lysis buffer (Promega, Madison, WI) according to the manufacturer's recommended protocol and luciferase activity was measured using a MicroLumat Plus LB96V luminometer (EG&G Berthold).

Luciferase activity was normalized to β-galactosidase activity measured as described by Sambrook and Russell [21] to account for minor differences in transfection efficiency. Normalized data are expressed as the mean fold-change (±S.E.M.) relative to that measured in vehicle-treated cells transfected with empty expression vector alone. All treatment transfections were performed in triplicate and each experiment was repeated at least three times. Data were subjected to ANOVA followed by a Fisher's LSD post hoc test ($p < 0.05$).

2.4. Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed using a modification of the procedure described by Lanzino et al. [22]. Briefly, PC-3(AR) cells were seeded in 6-well plates and treated with 10 nM DHT and/or 10 nM TCDD or vehicle for 30 min. The cells were washed twice with ice-cold 1× phosphate-buffered saline (PBS, pH 7.4) after fixation in 1% formaldehyde at 37 °C for 10 min. The cells were then collected and resuspended in 200 μl ice-cold lysis buffer [22] and centrifuged at 650 × *g* for 10 min at 4 °C. The pellets were resuspended in 250 μl of ice-cold lysis buffer containing complete protease inhibitor cocktail and incubated on ice for 30 min. Cell nuclei were lysed by passing cell lysates through a syringe (5 times), followed by DNA shearing by sonication. The lysates were then centrifuged at 20,500 × *g* for 12 min at 4 °C. The resulting supernatants were diluted in 45 μl of ChIP buffer [22] and the chromatin was precleared by incubation with 15 μl Protein A-Sepharose 4B and 2 μg salmon sperm DNA (Invitrogen) with rotation for 2 h at 4 °C. A volume (10 μl) of the precleared chromatin (input) was collected and the remainder was immunoprecipitated with 5 μg of anti-AhR antibody, or anti-AR antibody (used as a positive control) or normal rabbit IgG (used as a negative control) overnight at 4 °C with rotation. A volume of Protein A-Sepharose 4B (10 μl) and salmon sperm DNA (2 μg) were then added to each tube and incubated for further 2 h at 4 °C with rotation. The beads with antibody/chromatin complex were washed sequentially for 5 min each with wash buffer A, B, and C (as described by Lanzino et al. [22]) and then twice with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The immune complexes were eluted by addition of 50 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) and incubated at room temperature for 15 min with gentle shaking and the eluates were collected by centrifugation. A volume of 5 M NaCl (4 μl) and 10 mg/ml RNase A (1 μl) were added to each tube and to the input aliquots, and were reversed cross-linked by incubation at 65 °C overnight. The tubes were then incubated at 42 °C for 2 h after addition of 2 μl 0.5 M EDTA, 2 μl Tris–HCl pH 6.7 and 2 μl of 10 mg/ml proteinase K. DNA was obtained by phenol–chloroform extraction and PCR was performed using primers targeting the androgen response element II (AREII) of the PSA promoter using 5′ GGGATCAGGGAGTCTCACAA 3′ as forward and 5′ GGACAAAG-