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Insulin like growth factor 2 regulation of aryl hydrocarbon receptor in MCF-7 breast cancer cells

Justin K. Tomblin, Travis B. Salisbury *

Department of Pharmacology, Physiology and Toxicology, Joan C. Edwards School of Medicine, Marshall University, 1 John Marshall Drive, Huntington, WV 25755, USA

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ABSTRACT

Insulin like growth factor (IGF)-1 and IGF-2 stimulate normal growth, development and breast cancer cell proliferation. Cyclin D1 (CCND1) promotes cell cycle by inhibiting retinoblastoma protein (RB1). The aryl hydrocarbon receptor (AHR) is a major xenobiotic receptor that also regulates cell cycle. The purpose of this study was to investigate whether IGF-2 promotes MCF-7 breast cancer proliferation by inducing AHR. Western blot and quantitative real time PCR (Q-PCR) analysis revealed that IGF-2 induced an approximately 2-fold increase ($P < .001$) in the expression of AHR and CCND1. Chromatin immunoprecipitation (ChIP), followed by Q-PCR indicated that IGF-2 promoted ($P < .001$) a 7-fold increase in AHR binding on the CCND1 promoter. AHR knockdown significantly ($P < .001$) inhibited IGF-2 stimulated increases in CCND1 mRNA and protein. AHR knockdown cells were less ($P < .001$) responsive to the proliferative effects of IGF-2 than control cells. Collectively, our findings have revealed a new regulatory mechanism by which IGF-2 induction of AHR promotes the expression of CCND1 and the proliferation of MCF-7 cells. This previously uncharacterized pathway could be important for the proliferation of IGF responsive cancer cells that also express AHR.

1. Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor whose activity is regulated by lipid soluble environmental toxicants [1]. 2,3,7,8 Tetrachlorodibenzo-p-dioxin (TCDD) is a prototypical AHR agonist which is found in Agent Orange [1]. The binding of TCDD to AHR stimulates the AHR to translocate into the nucleus and stimulate transcription through specific xenobiotic response elements (XREs) in enhancers and promoters of TCDD stimulated genes [1,2]. TCDD through AHR induces the expression of a "battery" of phase I and phase II drug metabolizing enzymes including the prototype TCDD–AHR gene target cytochrome P450, family 1, subfamily A, polypeptide 1 (CYP1A1) [1,2].

The AHR also regulates cell cycle in part by binding with Cyclin D1 (CCND1) and cyclin dependent kinase 4 (CDK4) [3,4]. CDK4 phosphorylates retinoblastoma protein 1 (RB1), which inhibits RB1-mediated repression of E2F transcription factors [5–7]. The activation of E2F induces the expression of E2F target genes that are important for DNA synthesis and cell cycle advance [5–7]. Mitogens promote CDK4 activity by increasing the levels of cyclin proteins including CCND1 [5–7]. By functioning as a regulatory subunit on CDK holoenzymes, CCND1 promotes the phosphorylation and inhibition of RB1 to promote cell cycle advance and proliferation [5–7]. The AHR binds to CDK4 during advance through the cell cycle in human MCF-7 breast cancer cells [4]. TCDD binding to AHR attenuates AHR binding with CDK4, which correlated with cell cycle arrest and reductions in RB1 phosphorylation in MCF-7 cells [4]. CCND1 was also present in CDK4-AHR complexes [4].

Insulin like growth factor (IGF)-1 and IGF-2 stimulate growth, development and the proliferation of human cancer cells including breast cancer cells [8,9]. MCF-7 breast cancer cells have been reported to express high levels of IGF-1 receptor (IGF-1R) and insulin receptor subtype A receptor (IR-A) [8,9]. IGF-R1 and IR-A mediate the proliferative effects of IGFs on human breast cancer cells by inducing the phosphoinositide 3-kinase (PI3K)/AKT (protein kinase B) pathway and the mitogen-activated protein kinase (MAPK) pathway [8–10]. IGF-1 and IGF-2 have also been reported to increase levels of CCND1 to induce proliferation [6,8,9]. CCND1 promoter activity is regulated through multiple enhancers including activator protein-1 (AP-1) and T-cell factor-1 (Tcf-1)/lymphoid enhancing factor-1 (Lef-1) sites [11–14]. The transcription factors Jun and Fos bind to the AP-1 response elements [11,12]. The transcriptional co-activator β-catenin confers transcriptional activity to TCF/LEF transcription factors bound to TCF/LEF elements in the CCND1 promoter [13,14].

We have recently shown that adipocytes secrete levels of IGF-2 that are sufficient to stimulate the proliferation of MCF-7 and T-47D breast cancer cells [15]. We also found that AHR knockdown

* Corresponding author. Fax: +1 304/696 7391.
E-mail address: salisburyt@marshall.edu (T.B. Salisbury).

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MCF-7 cells were less responsive to the proliferative effects of IGF-2 [15]. The purpose of this study was to investigate if: (1) IGF-2 signaling regulates the AHR and (2) IGF-2 induction of CCND1 requires AHR. We provide evidence that IGF-2 signaling activates AHR and that AHR is important for inducing the expression of CCND1 and MCF-7 proliferation. This is a new link between IGF-2 signaling and AHR.

2. Methods

2.1. Materials and MCF-7 cell culture

Dulbecco's Modified Eagle Medium/High glucose (DMEM) with L-glutamine and sodium pyruvate, 10% fetal bovine serum, penicillin, and streptomycin (100 μg/mL) and phosphate buffered saline (PBS) were purchased from Fisher Scientific. Non-specific control RNA (cRNAi) (cat # D-001810-01-20), short interfering RNA against the AHR (AHRi) (J-004990-08-0010) and Dharmafect transfection reagent (#1) were purchased from Thermo Scientific. MCF-7 human breast cancer cells were purchased from ATCC (Manassas) and maintained in DMEM, 10% FBS, with penicillin (100 U/mL) and streptomycin (100 μg/mL) and .01 μg/mL bovine insulin (Cell Applications, Inc.) Insulin like growth factor 2 (IGF-2) was purchased from R & D systems and reconstituted in phosphate buffered solution.

2.2. Western blot analysis to determine IGF-2 induction of AHR and CCND1

200,000 MCF-7 cells plated in 35 mm plates (50% confluent) were serum starved overnight in phenol red-free DMEM and then treated with PBS vehicle or IGF-2 (100 ng/mL) for 3 h. This time point was selected based on our preliminary time course experiments showing that IGF-2 induction of CCND1 mRNA is maximal at 3 h post IGF-2 (data not shown). Total cellular extract was isolated in 200 μL of 2× sample lysis buffer (Bio-RAD; cat #161-0737) and approximately 12.5 μg of protein was subjected to SDS PAGE and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad; Hercules, CA). Membranes were blocked in PBS, .01% Tween 20 (Bio-Rad; Hercules, CA) (PBS-T), 5% (wt/vol) lowfat powdered milk for 1 h and incubated overnight with primary antibody at 4 °C with gentle mixing. Membranes were rinsed five times (five minutes each wash) with PBS-T and then incubated with an appropriate HRP-labeled secondary antibody (diluted 1:10,000 in PBS, .01% tween-20, 5% milk) (Thermo Scientific, Pierce) for 1 h, followed with rinsing five times (five minutes each wash) in PBS-T. Membranes were developed with enhanced chemiluminescent substrate (Millipore, Immobilon ECL substrate) and exposure to X-ray film (Midwest Scientific). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) antibody was purchased from Millipore (cat # MAB374), AHR antibody from Santa Cruz (Cat # H-211) and CCND1 antibody from Millipore (cat # 04-1151). Equal protein loading was confirmed by GAPDH Western blots. Normalized levels of AHR, and CCND1 were expressed as a ratio of AHR/GAPDH and CCND1/GAPDH. Densitometry was calculated with ImageJ PC-based software (National Institute of Health).

2.3. Q-PCR analysis to determine IGF-2 induction of AHR, CYP1A1 and CCND1

200,000 MCF-7 cells plated in 35 mm plates were serum starved overnight in phenol red-free DMEM, and then treated with PBS vehicle or IGF-2 (100 ng/mL) for 3 h. Total RNA was isolated in TRI-Reagent and quantitated by Nanodrop spectrophotometry. RNA was reverse transcribed to complementary DNA (cDNA) (Verso cDNA kit; Thermo Fisher Scientific; cat # AB-1453/B). Resulting cDNAs were subjected to quantitative real-time PCR (Q-PCR) using gene specific primers (300 nM per reaction) and 40 cycles of PCR in accordance with Absolute Blue SYBR Green Rox Mix (Thermo Fisher Scientific; cat # AB-4162/B) protocols. Relative gene expression between controls and IGF-2 treated cells was calculated using the formula $2-\Delta\Delta CT$, as described by Livak and Schmittgen [16]. Glyceraldehyde-3-phosphate (GAPDH) mRNA levels served as the internal control. Primer sequences for GAPDH, AHR, CCND1 and CYP1A1 were: GAPDH (forward, 5′-CATGAGAAGTATG ACAACAGCCT-3′; reverse, 5′-AGTCCTTCCACGATACCAAAGT-3′), AHR (forward, 5′-ACATCACCTACGCCAGTGG-3′; reverse, 5′-CTCTAT GCCGCTTGGAAGGAT-3′), CCND1 (forward, 5′-CCGCAATGACCCCGC ACGAT-3′; reverse, 5′-AGGGCAACGAAGGTCTGCGC-3′) and CYP1A1 (forward, 5′-CTTCACCCTCATCAGTAATGGTC-3′; reverse, 5′-AGGCTG GGTCAGAGGCAAT′-3). The Harvard Primer Bank http://pga.mgh.harvard.edu/primerbank/ was used to design primers. Primer specificity was verified with melt curve analysis and NIH primer blast search engines http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome.

2.4. Chromatin immunoprecipitation

MCF-7 cells (500,000 per 60 mm plate) were serum starved overnight in phenol red-free DMEM, and then treated with PBS vehicle or IGF-2 (100 ng/mL) for 3 h. Formaldehyde (1%) was then added to medium for 10 min, followed by Glycine (.5 M) for 5 min. Cells were rinsed with PBS, collected in PBS, pelleted by centrifugation, and lysed in 300 μL of lysis buffer (1% SDS; 5 mM EDTA; 50 mM Tris–HCl, pH 8) per 60 mm plate plus protease inhibitors (Thermo Scientific) for 15 min on ice. Cell extracts were sonicated (5 times, each time 10 s) and diluted 1:10 in dilution buffer (16.7 mM Tris–HCl, pH 8; 167 mM NaCl; 1.2 mM EDTA; 0.01% SDS; 1.1% Triton X-100), rotated overnight at 4 °C with 1 μg of non-specific IgG (Santa Cruz; cat # 2027) or anti-AHR antibody (Santa cruz; cat # H-211). Antibody-chromatin complexes were collected using 5 μL of magnetic protein A beads (life technologies; cat # 100.01D) with rotation at 4 °C for 90 min. Using magnetic separation (life-technologies; part # 49-2025), beads were washed sequentially with buffer 1 (20 mM Tris–HCl, pH 8; 150 mM NaCl; 2.0 mM EDTA; 0.1% SDS), buffer 2 (20 mM Tris–HCl, pH 8; 500 mM NaCl; 2.0 mM EDTA; 0.1% SDS), buffer 3 (10 mM Tris–HCl (pH 8); 0.25 M LiCl; 1 mM EDTA; 1% NP-40; 1% deoxycholate), and then 1× TE buffer for five minutes each, and incubated at 65 °C for 4–6 h in elution buffer (1% SDS, 0.1 M $NaHCO_3$) with proteinase K. DNA was purified (Qiagen; cat # 28204) and analyzed using real time PCR. Primers spanning AP-1 and TCF/LEF response elements in the CCND1 promoter and xenobiotic response elements (XREs) in the CYP1A1 promoter were used: AP-1 (forward, 5′-GGCAGAG GGGACTAATATTTCCAGCA-3′; reverse, 5′-GAATGGAAAGCTGAGAA ACAGTGATCTCC-3′) [17], TCF/LEF (forward, 5′-GCTCCCATTCTCTGC CGG-3′; reverse, 5′-CGGAGCGTGCGGACTCTG-3′) [18] and XRE (forward, 5′-ACGCAGACCTAGACCCTTTGC-3′, reverse, 5′-CGGGTGCGC GATTGAA-3′) [19]. ChiP data was expressed as % input, in which signals obtained from the ChIP are divided by signals obtained from an input sample.

2.5. AHR knockdown experiments

MCF-7 cells were reverse transfected using methods we have used previously to selectively target genes for knockdown [20]. Briefly, 200,000 MCF-7 cells were plated in phenol red-free DMEM, 5% charcoal treated FBS, 50 nM cRNAi or AHRi, 2 μL of Dharmafect #1 per well of a 6 well plate for 12 h and then new media was applied for 24 h. Cells were then serum starved overnight in phenol