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Overexpression of ERβ is sufficient to inhibit hypoxia-inducible factor-1 transactivation

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ABSTRACT

Estrogen receptor (ER) β is predicted to play an important role in the prevention of breast cancer development and progression. We have previously shown that ERβ suppresses hypoxia inducible factor (HIF)-1-mediated transcription through aryl hydrocarbon receptor nuclear translocator (ARNT) degradation via ubiquitination processes. In this study, we attempted to examine the effect of ERβ specific ligand on HIF-1 inhibition in ERβ positive PC3 cells and ERβ transfected MCF-7 cells. ERβ specific agonist diarylpropionitrile (DPN) stimulated estrogen response element (ERE)-luciferase activity in a similar fashion to estradiol in PC3 cells. We observed that DPN down-regulates the ARNT protein levels leading to an attenuation of hypoxia-induced hypoxia response element (HRE)-driven luciferase reporter gene activation in PC3 cells. Treatment of DPN reduced vascular endothelial growth factor (VEGF) expression and co-treatment with ERβ specific antagonist PHTPP abrogated the effect in PC3 cells. We then examined the effect of DPN in ERβ transfected MCF-7 cells. HIF-1 transcriptional activity repression by ERβ was not further reduced by DPN, as examined by HRE-driven luciferase assays. Expression of ERβ significantly decreased VEGF secretion and ARNT expression under hypoxic conditions. However, DPN did not additionally affect this suppression in MCF-7 cells transfected with ERβ. This result shows that unliganded ERβ is sufficient to inhibit HIF-1 in systems of overexpression.

1. Introduction

Estrogen plays an important role in many non-reproductive tissues, including the immune, cardiovascular and central nervous systems, as well as in reproductive tissues, such as the breast, ovary, and uterus [1]. The cellular response to estrogen is mediated by two estrogen receptor (ER) isoforms, ERα and ERβ, which belong to the family of nuclear receptors [2]. A series of reports assert that in contrast to ERα, which is an activator of cancer cell growth, ERβ, when present together with ERα, has a generally restraining effect on ERα activities [3,4]. ERβ expression attenuates the growth-promoting activity of ERα [3]. The varying intracellular ERα/ERβ ratio affects the estrogen-induced cell proliferation [5]. In addition to its role in modulating ERα-mediated regulation, ERβ also has distinct functions. Expression of ERβ inhibits cancer cell growth and prevents tumor expansion by inhibiting angiogenesis [6]. ERβ inhibits the proliferation and tumor growth of colon cancer cells [7] and sustains epithelial differentiation [8]. ERβ destabilized epidermal growth factor receptor (EGFR) and inhibited epithelial to mesenchymal transition (EMT) of basal-like breast cancer cells. It was suggested that ERβ might be a crucial marker in the prediction of breast cancer [9].

Hypoxia is a state of reduced overall tissue oxygen availability and causes many pathological states, including ischemic disease, chronic inflammatory disease and cancer [10]. Activation of hypoxia-inducible factor (HIF) proteins in response to hypoxia stimulates a transcriptional program that promotes angiogenesis and apoptosis [11–13]. HIF factors are heterodimeric proteins consisting of a HIF-1α subunit and a constitutively expressed HIF-1β subunit, also referred to as aryl hydrocarbon receptor nuclear translocator (ARNT) [13]. Under hypoxic conditions, HIF-1α is stabilized, and regulates transcription by binding to the hypoxia response element (HRE) on target genes [14]. Recruitment of ARNT is critical to enable HIF-1α to bind to HREs, further illustrating the central role of ARNT in regulation of HIF-1α function [15]. Moreover, ARNT directly interacts with ERs and has been shown by transient transfection to co-activate ER-dependent gene expression [2,16]. The regulation of ARNT is implicated to have a significant impact on hypoxia and estrogen signaling pathways. Hypoxia is a hallmark of solid tumor, which leads to cell invasion and metastasis [17]. HIF-1 transcriptional activity was proposed to be in part responsible for the enhanced invasive properties of cancer cells

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[18,19]. Several reports have indicated the association of ERβ with the regulation of EMT.

We recently reported that ERβ inhibits HIF-1α-mediated transcription by destabilizing ARNT [20]. However, the effect of ERβ-specific ligand on HIF-1 inhibition was not clear. This study focuses on whether ligand-activated ERβ is a prerequisite for HIF-1 inhibition. We show that HIF-1 suppression by ERβ is dependent of ligand in ERβ positive cells, but independent of ligand in systems overexpressing ERβ.

2. Material and methods

2.1. Materials

17-β-estradiol (E2) was purchased from Sigma (St. Louis, MO, USA) and dissolved in 100% ethanol. Diarylpropionitrile (DPN) and 4-[2-phenyl-5,7-bis(trifluoro methyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP) were obtained from Tocris (Bristol, UK) and dissolved in 100% ethanol. Fetal bovine serum (FBS) was purchased from WelGENE (Daegu, South Korea). Trizol Reagent and penicillin/streptomycin were purchased from GIBCO Invitrogen (Grand Island, NY, USA). Anti-ARNT and anti-HIF-1α were obtained from BD Biosciences (San Jose, CA, USA). Anti-β-actin and anti-Flag were purchased from Sigma (St. Louis, MO, USA). Anti-HA was kindly provided by Dr. Yong-Hee Lee (Chungbuk National University, Korea).

2.2. Cell culture and hypoxic conditions

PC3 [21] and A549 cells [22] were maintained in phenol red-free Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and penicillin/streptomycin. MCF-7 cells [4] were maintained in phenol red-free RPMI 1640 medium supplemented with 10% FBS and penicillin/streptomycin. Cells were grown at 37 °C in a humidified atmosphere of 95% air/5% CO_2 and fed every 2–3 days. Before treatment, the cells were washed with phosphate-buffered saline and cultured in DMEM/5% charcoal–dextran stripped FBS (CD-FBS) for 2 days. All treatments were done with DMEM or RPMI 1640/5% CD-FBS. For the hypoxic condition, cells were incubated at a CO_2 level of 5% with 1% O_2 balanced with N_2 using a hypoxic chamber (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Plasmids

The ERE2-tk81-luc, constructed by inserting the fragment of the herpes simplex thymidine kinase promoter and two copies of the vitellogenin ERE into pA3luc (ERE-luc), was a kind gift from Dr. Larry Jameson. The HRE-Luc reporter plasmid contains four copies of the erythropoietin HRE, the SV40 promoter, and the luciferase gene. Flag-ERβ expression vector was kindly provided by Dr. Mesut Muyan (University of Rochester Medical School, USA).

2.4. Transfection and luciferase assays

PC3, A549 and MCF-7 cells were transiently transfected with plasmids by using the polyethylenimine (PEI; Polysciences, Warrington, PA, USA). Luciferase activity was determined 24 or 48 h after treatment with an AutoLumat LB9507 luminometer (EG & G Berthold, Bad Widbad, Germany) using the luciferase assay system (Promega Corp., Madison, WI, USA) and expressed as relative light units.

PC3 cells were transfected transiently with Lipofectamine 2000 (Invitrogen) and On-Target Plus SMARTpool siRNAs (Dharmacon, Lafayette, CO, USA) for ERβ twice with a 24 h time interval for maximum efficiency. These target sequences have been published by Dharmacon. Nontargeting pools were used as negative controls.

2.5. Quantitative real-time polymerase chain reaction (PCR)

Total RNA was extracted using Trizol Reagent according to the manufacturer's instruction. Quantitative real-time PCR (qPCR) was performed using iQ™ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). The primers used were: β-actin sense primer, 5′-CAAATGCTTCTAGGCGGACTATG-3′; β-actin anti-sense primer, 5′-TGCGCAAGTTAGGTTTTGTCA-3′; vascular endothelial growth factor (VEGF) sense primer, 5′-CTGCTGTCTTGGGTGCATTGG-3′; VEGF anti-sense primer, 5′-GTTTGATCCGCATAATCTGCAT-3′. A final volume was 25 μl, and an iCycler iQ Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) was used for qPCR. The amplification data were analyzed by iQ™5 optical system software version 2.1 and calculated using the $\Delta\Delta C_T$ method. The $\Delta\Delta C_T$ method was used to calculate relative mRNA expression.

2.6. VEGF ELISA

After hypoxic exposure, culture medium was removed and stored at −80 °C until assayed. VEGF concentrations were determined using ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Samples from two different experiments were analyzed in triplicate.

2.7. Western blot analysis

Protein was isolated in lysis buffer (150 mM NaCl, 50 mM Tris–HCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% deoxycholate, 1% SDS) with protease inhibitor cocktail (Sigma, St. Louis, MO, USA) on ice for 1 h and then centrifuged for 20 min at 13,000×*g*. Supernatant was collected and protein concentrations were measured using the Bradford method (Bio-Rad, Hercules, CA, USA). Proteins were dissolved in sample buffer and boiled for 5 min prior to loading onto an acrylamide gel. After SDS–PAGE, proteins were transferred to a polyvinylidene difluoride membrane, blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 60 min at room temperature. The membranes were incubated for 2 h at room temperature with antibody. Equal lane loading was assessed using β-actin monoclonal antibody (Sigma, St. Louis, MO, USA). After washing with TBST, blots were incubated with 1:5000 dilution of the horseradish peroxidase conjugated-secondary antibody (Invitrogen, Grand Island, NY, USA), and washed again three times with TBST. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.8. Immunoprecipitation

Two hundred microgram of the cell lysates were mixed with 1 μg of antibody and incubated overnight at 4 °C with constant rotation. To recover immunoprecipitated complexes, 150 μl of protein A-sepharose, diluted 1:1 in PBS, were then added to the samples and incubated on ice for additional 2–4 h with constant rotation. The beads were pelleted by centrifugation and the eluted proteins were analyzed by immunoblot analysis.

2.9. Cell invasion assays

The invasion assay was performed with Transwell inserts that have 6.5-mm polycarbonate membranes with pores 8.0-μm in size. Matrigel invasion assay was performed using membranes coated with Matrigel matrix (BD Science, Sparks, MD, USA). PC3 and A549 cells were seeded into the upper chamber in serum-free

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