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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 [2]. The cellular response to estrogen is mediated by two estrogen receptor (ER) isoforms, ERα and ERβ, which belong to the family of nuclear receptors [3]. 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 [4,4]. ERβ expression attenuates the growth-promoting activity of ERα [4]. The varying intracellular ERα/ERβ ratio affects the estrogen-induced cell proliferation [6]. 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 [7]. ERβ inhibits the proliferation and tumor growth of colon cancer cells [8] and sustains epithelial differentiation [9]. 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 [10].

Hypoxia is a state of reduced overall tissue oxygen availability and causes many pathological states, including ischemic disease, chronic inflammatory disease and cancer [11]. Activation of hypoxia-inducible factor (HIF) proteins in response to hypoxia stimulates a transcriptional program that promotes angiogenesis and apoptosis [12–13]. 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) [14]. Under hypoxic conditions, HIF-1α is stabilized, and regulates transcription by binding to the hypoxia response element (HRE) on target genes [15]. 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 [16]. Moreover, ARNT directly interacts with ERs and has been shown by transient transfection to co-activate ER-dependent gene expression [3,16]. 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 [18]. HIF-1 transcriptional activity was proposed to be in part

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responsible for the enhanced invasive properties of cancer cells [19,19]. 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 [21]. 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 [22] and A549 cells [23] were maintained in phenol red-free Dulbecco's modified Eagle medium (DMEM) supplemented with 10% FBS and penicillin/streptomycin. MCF-7 cells [5] 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

media. The lower chambers consisted of DMEM media containing 10% FBS. After incubation under normoxia or hypoxia for 48 h, non-invasive cells present on the upper surface of the membrane were scraped with cotton swabs and the invasive cells present on the lower side of the membrane were fixed with ice cold methanol, stained with 0.1% crystal violet. Five randomly selected fields on the fixed Transwell chamber were captured by photography, and invaded cells were counted.

2.10. Statistical analysis

Data were expressed as means ± SD, and statistical analysis for single comparison was performed using the Student's *t* test. The criterion for statistical significance was $p < 0.05$.

3. Results

3.1. Ligand-occupied ERβ inhibit HIF-1 pathway in ERβ-positive PC3 cells

We have previously shown that ERβ suppresses HIF-1α-mediated transcription via ARNT downregulation. In this study, we aimed to examine the effect of ERβ-specific ligand on HIF-1 inhibition. First, we studied the effect of DPN-occupied ERβ on HIF-1α-mediated gene transcription by using a HRE-driven reporter gene. PC3 human prostate cancer cells, which are known to express endogenous ERβ, were transfected with an HRE-Luc plasmid without an expression vector for ERβ under conditions of hypoxia. As shown in Fig. 1A, the HRE-driven luciferase reporter was markedly activated by hypoxia, and DPN significantly inhibited this hypoxic activation. Second, in order to confirm that the activities of DPN were ERβ mediated, we co-incubated the cells with the ERβ specific antagonist PHTPP, at a concentration which was sufficient to inhibit ERβ in the cells (10 μM). Suppression of hypoxic induction of VEGF by DPN was restored by PTHPP, verifying that the activities are ERβ-mediated (Fig. 1B). Third, DPN treatment under hypoxia downregulates the ARNT protein level in PC3 cells in the absence of ERβ transfection (Fig. 1C). These results imply that ligand occupied endogenous ERβ is functional in HIF-1 transrepression attributed to the downregulation of ARNT. The involvement of ERβ modulation of ARNT protein level was also confirmed after knockdown of ERβ using RNA interference in PC3 cells. As shown (Fig. 1D), VEGF mRNA levels were increased when the expression of ERβ was repressed in PC3 cells. Knockdown of ERβ mRNA by ERβ-siRNA were validated by qPCR.

3.2. Overexpression of ERβ is sufficient to inhibit the transactivation of HIF-1

In order to examine the effect of ERβ ligand under the ERβ overexpressed state, MCF-7 cells were transfected with an HRE-Luc plasmid with an expression vector for ERβ under hypoxia, and were treated with DPN. As shown in Fig. 2A, the HRE-driven luciferase reporter was markedly activated by hypoxia. Consistent with previous reports, ERβ significantly inhibited this hypoxic activation. However, no further inhibition was observed with DPN treatment. We then examined the effect of hypoxia-induced VEGF secretion. MCF-7 cells were transfected with vector control or ERβ, exposed to hypoxia for 24 h, and treated with DPN. As shown in Fig. 2B, the expression of ERβ significantly decreased VEGF secretion under hypoxic condition but no further decrease with DPN. In addition, DPN treatment was not effective in the ERβ

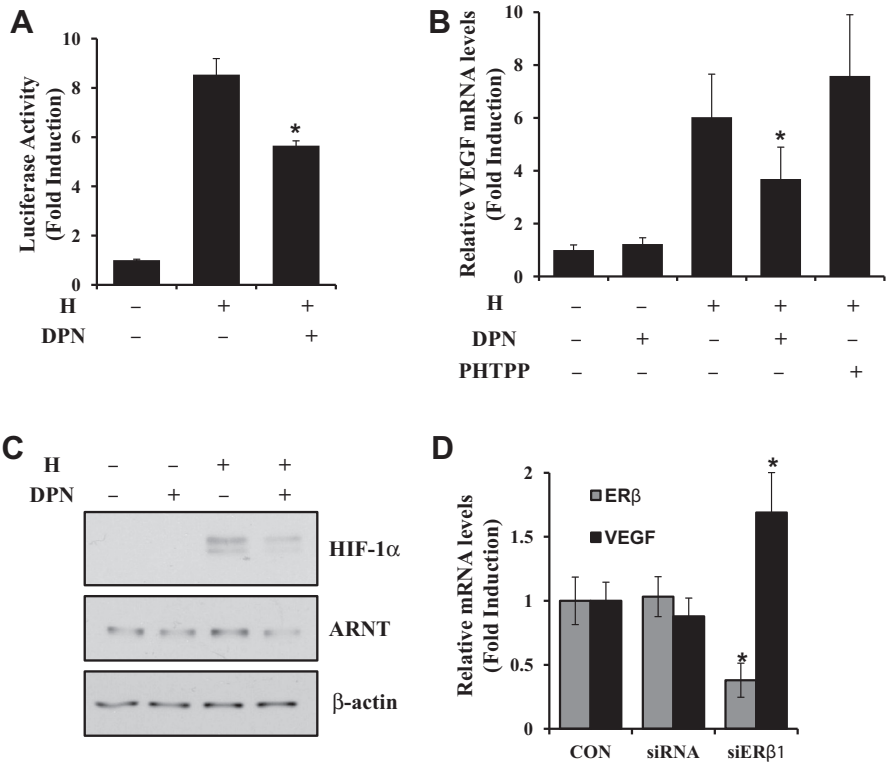

Fig. 1. DPN represses HIF-1 in ERβ positive PC3 cells. (A) PC3 cells were transfected with HRE-Luc reporter were left untreated or treated with 1 μM DPN and incubated for 24 h under normoxic or hypoxic conditions. Luciferase expression values represent the mean ± S.D. ($N = 3$). $^*p < 0.05$. (B) PC3 cells were treated for 24 h with 1 μM DPN and/or 10 μM PHTPP. Expression of VEGF was analyzed by qPCR. $^*p < 0.05$. (C) PC3 cells were treated for 24 h with 1 μM DPN with or without hypoxia. Total protein extracts were immunoblotted as indicated. (D) PC3 cells were twice transfected with control siRNA or ERβ SMARTpool siRNA. Expression of VEGF and ERβ was analyzed by qPCR. $^*p < 0.05$. Shown are representative results from 2 to 3 separate experiments.

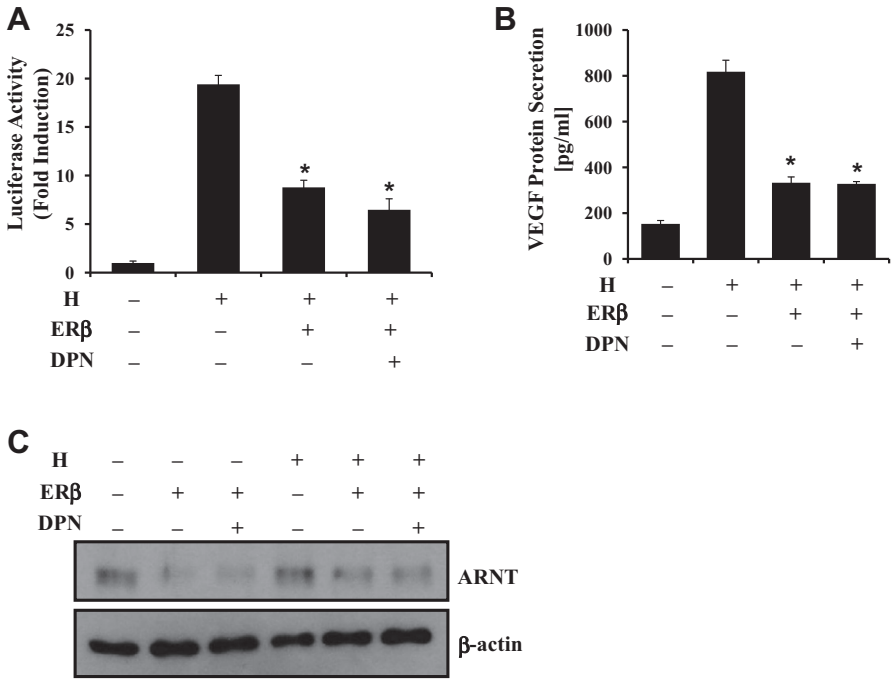

Fig. 2. ERβ decreases HIF-1 mediated gene transcription but ERβ agonist had no additional effect. (a) MCF-7 cells were cotransfected with HRE-Luc reporter and ERβ. Cells were left untreated or treated with 1 nM DPN for 24 h under hypoxic conditions, and luciferase expression was determined. $^{*}p < 0.05$. (B, C) MCF-7 were transfected with ERβ, treated with 1 nM DPN and incubated for 24 h under hypoxic conditions, and analyzed using VEGF ELISA kit (B) or immunoblotted as indicated (C). Shown are representative results from 2 to 3 separate experiments.

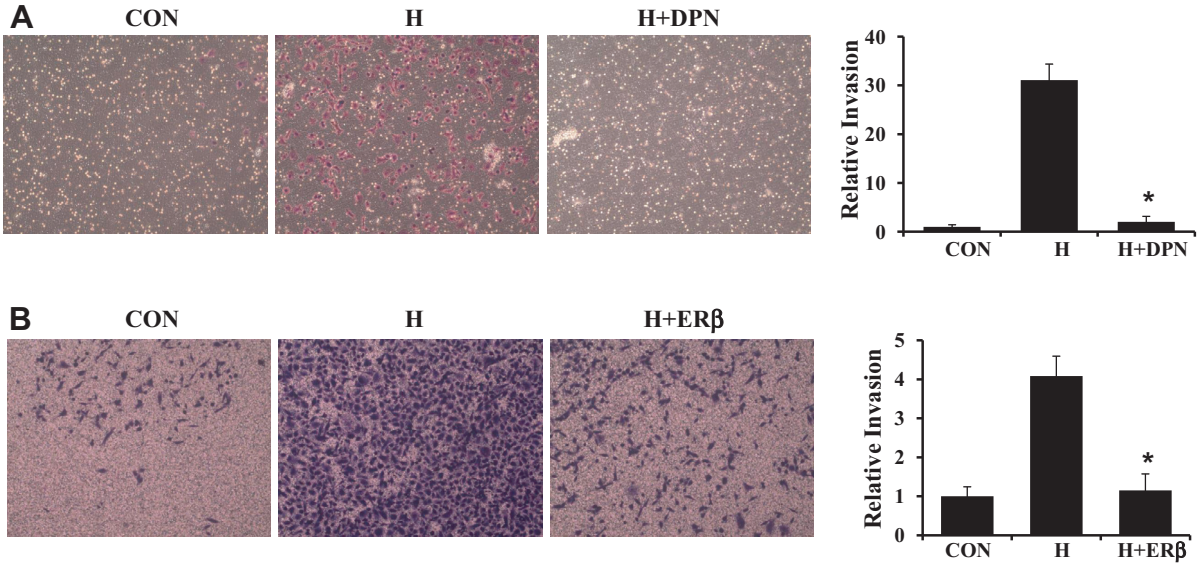

Fig. 3. ERβ inhibits cell invasion under hypoxia. (A) PC3 cells were untreated or treated with 1 μM DPN and incubated for 48 h under hypoxic conditions assessed for invasion by using Matrigel-coated Transwell chambers. The cells that were translocated to the lower surface of the filter were shown. $^{*}p < 0.05$. (B) Control (pcDNA 3.1) and ERβ-expressing A549 cells were incubated for 48 h under normoxic or hypoxic conditions assessed for invasion. The cells that were translocated to the lower surface of the filter were shown. $^{*}p < 0.05$. Shown are representative results from 2 separate experiments.

induced degradation of ARNT (Fig. 2C). These results show that unoccupied ERβ itself serves as a negative regulator of HIF-1 under ERβ overexpressed conditions.

3.3. ERβ attenuates hypoxia-induced invasion

The hypoxic microenvironment within solid tumors has been increasingly recognized as an important driver of local invasion [18]. To decipher the biological relevance of reduced HIF-1 expression by ERβ, we examined the effect of DPN treated on PC3 cells and ERβ over-expression on A549 cell invasion capability under hypoxia. We investigated the invasion ability of DPN treated PC3 cells by Matrigel invasion assay. Hypoxia exposure increased PC3 cell invasion. DPN decreased PC3 cell invasion induced by hypoxia (Fig. 3A). We attempted to use MCF-7 cells but the invasion was not significant under hypoxia. A549 cells were used instead.

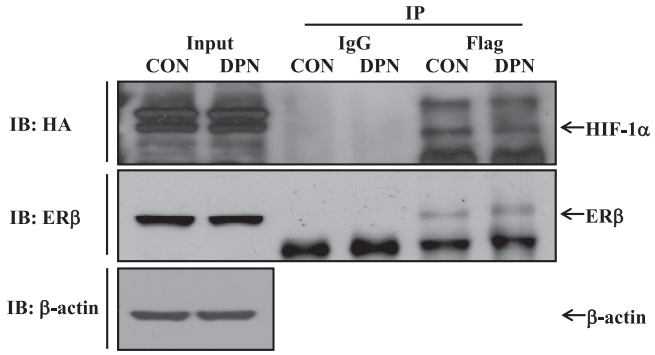

Fig. 4. ERβ interacts with HIF-1α. MCF-7 cells were cotransfected with HA-HIF-1α and Flag-ERβ. Cells were left untreated or treated with DPN under hypoxic conditions. Cell lysates were immunoprecipitated with anti-Flag antibody and precipitated proteins were analyzed by immunoblots with anti-HA (top) or anti-ERβ antibody (bottom). Shown are representative results from 3 separate experiments.

As shown in Fig. 3B, invading capability in A549 cells were increased under hypoxia as compared with those under normoxia. ERβ also inhibited the invasion of A549 cells under hypoxic conditions by 70% (Fig. 3B). These findings indicate that ERβ has anti-invasive properties under a hypoxic microenvironment.

3.4. ERβ interacts with HIF-1α under hypoxia

Previous data imply the interaction between HIF-1α and ERβ, and prompted us to investigate the possibility that ERβ might interact with HIF-1α. We examined whether ARNT degradation occurred in the presence of an interaction between ERβ and the HIF-1α. MCF-7 cells were co-transfected with HA-HIF-1α and Flag-ERβ expression vectors, and were incubated under hypoxia. As shown in Fig. 4, an interaction between HIF-1α and ERβ was detected upon cotransfection with HA-HIF-1α and Flag-ERβ under hypoxia. In addition, interaction between HIF-1α and ERβ were unaffected by ERβ ligand. These data suggested that the full length HIF-1α co-immunoprecipitated with ERβ.

4. Discussion

We and others have reported that ERβ inhibits HIF-1 signaling by destabilizing HIF-1α and ARNT in PC3 cells where endogenous ERβ is expressed [22], and in MCF-7 cells with ectopically expressed ERβ [21]. In this study, we compared the effect of ERβ specific ligand in obstruction of the HIF-1 pathway in both PC3 and MCF-7 cells. Interestingly, the overexpression of ERβ is sufficient to block the HIF-1 pathway and overcomes the need for the ligand. Our speculation is that the association of ERβ and HIF-1 recruits ubiquitin ligase components to the complex that induce the destabilization of HIF-1 complex. Overexpression of ERβ probably recruits sufficient concentrations of co-regulators in the vicinity of HIF-1 complex, whereas endogenous ERβ needs to be activated by ligand occupation for recruitment. Our previous results show that ERα is degraded by HIF-1α. However, ERβ degradation was not observed (our unpublished results). These results imply that HIF-1 and ER complexes contain ubiquitin ligases, although the substrate may differ depending on circumstances. The E3 ligase activity of Mdm2 promotes the ubiquitination and degradation of p53 and ERα [24]. Under hypoxia, HIF-1α is known to stabilize p53 through Mdm2-bridged interaction [25]. However, another report has shown a direct binding of p53 to the oxygen-dependent domain of HIF-1α [26]. Saji et al. [27] showed that Mdm2 directly interacts with ER, enhancing ER transactivation and degradation.

Hypoxia is a hallmark of solid tumor that leads to cell invasion and metastasis [18]. HIF-1 transcriptional activity was proposed to be, in part, responsible for the enhanced invasive properties of cancer cells [18,19]. HIF-1α plays an important role in embryonic stem cells (ESCs) differentiation which is regulated by EMT under hypoxia [28,28]. Overexpression or activation of ERβ significantly inhibited hypoxia-induced cell invasion, however, the pathway leading to this suppression is not fully known. Suppression of inflammation inhibits metastasis and delays tumor onset of poorly differentiated metastatic cancer cells [30,30]. It is possible that ERβ under hypoxia influences the inflammatory pathway, leading to an inhibition of metastasis.

Our results demonstrate that ERβ interacts with HIF-1α and is coupled to ARNT degradation under hypoxia. In parallel, previous data has shown that C-terminal domain of ERα interacted with the N-terminal bHLH/PAS domain of HIF-1α and was coupled to ERα degradation under hypoxia. However, how these interactions lead to the degradation of interacting proteins remains to be determined. Based on these results, elucidation of HIF-1and ER complexes will greatly aid our understanding of estrogen-controlled hypoxic tumors. Our continuous search for the identification of components of functional HIF-1 and ER complexes will elucidate the interaction with HIF-1 and function of ERβ under hypoxia.

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