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Hypoxic activation of unoccupied estrogen-receptor-alpha is mediated by hypoxia-inducible factor-1 alpha

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Abstract

The estrogen receptor (ER) plays an important role in breast cancer development and progression. Hypoxia has been shown to modulate the level of ERα expression, which is intimately associated with the biology of breast carcinomas. However, the effect of hypoxia on ERα-mediated transactivation is largely unknown. In this report, we have examined ligand-independent transcriptional activation of ERα by hypoxia. The hypoxia-induced ERα-mediated transcriptional response was inhibited by the ER antagonist ICI 182,780 as determined by transient expression of ERα and ER-responsive reporter plasmids in the HEK 293 cells. Hypoxic activation of ERα was dependent on the increased expression of hypoxia-inducible factor-1α (HIF-1α), as examined in HEK 293 cells under conditions of normoxia. These results indicate that hypoxia activates ERα in a ligand-independent manner, possibly through the interaction between HIF-1α and ERα.

Keywords: Estrogen receptor alpha; Hypoxia; Hypoxia-inducible factor-1 alpha

1. Introduction

The estrogen receptor (ER) is an estrogen-activated transcription factor. On binding estrogen, the ER undergoes phosphorylation and conformational changes that induce tight binding to the cognate estrogen-responsive element and thereby modulates the transcription of target genes [1,2]. Ligand-occupied ER activity is affected by more than 30 co-regulatory proteins, including the steroid receptor co-activator, CREB-binding protein and aryl hydrocarbon receptor nuclear translocator (ARNT) [3,4]. Even in the absence of estrogen, the activity of the ER can be regulated by a number of other stimuli, such as growth factors, cAMP, dioxin, leptin and other hormone-bound steroid hormone receptors [5–8]. Studies indicate that phosphorylation of ERα at Ser 104, 106, 118 and 167 by cellular kinases, the acetylation state of the ER, and the recruitment of co-activators serve as mechanisms for ligand-independent activation [9,10]. While elucidation of the mechanisms that govern ER activation is crucial to understanding ER function, the precise details of the regulation remain unclear.

Hypoxia is important in normal physiological processes and development, as well as in tumorigenesis [11]. Hypoxia can stimulate systemic, cellular and local responses by increasing the efficiency of oxygen transport throughout the body, limiting oxidative metabolism, decreasing energy production and increasing vasculature in embryogenesis, wound healing or tumor growth, respectively [12]. The regulation of gene expression during hypoxia is *via* hypoxia inducible factor-1 (HIF-1), which binds to the *cis*-elements within target genes such as vascular endothelial growth factor, nitric oxide synthase, various glycolytic enzymes and erythropoietin [13]. Functional HIF-1 exists as a heterodimer, which comprises HIF-1α and ARNT subunits [13]. HIF-1 activation depends on the existence of HIF-1α, which is usually degraded under conditions of normoxia and stably expressed only under hypoxia [13]. ARNT, a partner protein of HIF-

Abbreviations: AhR, aryl hydrocarbon receptor; ARNT, AhR nuclear translocator; CREB, cyclic AMP-response element binding protein; DBD, DNA binding domain; ER, estrogen receptor; E2, 17-β-estradiol; ERE, estrogen response element; GFP, green fluorescent protein; HIF-1, hypoxia inducible factor-1; ICI, ICI 182,780; IGF-1, insulin-like growth factor-1

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1α, also functions as a dimerization partner protein for the aryl hydrocarbon receptor (AhR), which acts as a receptor for dioxin [14]. Independent studies have shown that ARNT and AhR function as co-activators of ER, which raises the possibility that HIF-1α also plays a role in ER transactivation [4,7].

ERα plays an important role in breast cancer development and progression by influencing genes and signaling pathways that are involved in cellular proliferation [15]. Hypoxia has been shown to modulate the level of ERα expression [16], an important factor in the natural history of breast cancer [17]. High levels of HIF-1α expression have been observed in ERα-positive breast cancer [18], and HIF-1α-positive tumors express significantly lower levels of ER [19]. Hypoxia induces the degradation of ERα within 6–12 h *via* a proteasome-dependent pathway [16], which involves HIF-1α expression and physical interaction between ERα and HIF-1α [20]. It is possible that hypoxia alters ERα-mediated signaling as a consequence of ERα degradation, which is important and necessary for tumorigenesis. However, the molecular mechanisms underlying ER-mediated transactivation by hypoxia remain largely unknown.

In this study, we have examined the effect of hypoxia and HIF-1α expression on the transcriptional activation of ERα. Hypoxic induction of functional transactivation of ERα was studied in transient transfection systems using the ERα and estrogen-responsive reporters in the HEK 293 cells. To investigate the effect of HIF-1α expression on ERα activation, we used hybrid molecule of HIF-1α and green florescent protein (GFP), which were designed to be stable under normoxia conditions. Our results demonstrate that hypoxia activates ERα in a ligand-independent manner and that HIF-1α expression is sufficient to increase the ERα-mediated transcription response of estrogen-responsive reporter plasmids.

2. Materials and methods

2.1. Reagents

E2 was purchased from Sigma. ICI was obtained from ZENECA Pharmaceuticals. ICI was dissolved in DMSO and E2 in 100% ethanol. All the compounds were added to the medium such that the total solvent concentration was never higher than 0.1%. An untreated group served as a control.

2.2. Plasmids

The ERE-tk81-Luc plasmid, constructed by inserting the fragment of the herpes simplex thymidine kinase promoter and two copies of the vitellogenin ERE into pA3-luc, was a gift from Dr. Larry Jameson [21]. The pcDNA3.1-hERα plasmid was constructed by inserting the fragment of the full length hERα into pcDNA3.1 (Invitrogen). The pGAL4-Luc was obtained from Dr. Benita Katzenellenbogen. HIF-1α/VP16 DNA fragment was generated according to the methods published by Vincent et al. [22]. The hybrid DNA contains DNA binding/dimerization domain (amino acids 1–390) of HIF-1α and VP16 transactivation domain. The insert was cloned into JDK vector [23]. The hypoxia responsive element (HRE)-Luc reporter plasmid contains four copies of the erythropoietin HRE, the SV40 promoter, and the luciferase gene. GFP-HIF-1α vector was kindly provided by Dr. Kyu-Won Kim (Seoul National University, Korea).

2.3. Cell culture and hypoxic conditions

MCF-7 and HEK 293 cells were maintained in phenol red-free DMEM containing supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories). Cells were grown at 37 °C in a humidified atmosphere of 95% air/5% CO_2 and fed every 2–3 days. Before treatment with chemicals, the cells were washed with PBS and cultured in DMEM/5% charcoal–dextran stripped FBS (CD-FBS) for 2 days to eliminate any estrogenic source before treatment. All treatments were done with DMEM/5% CD-FBS. Ten nanomolar E2 has been used unless otherwise noted. 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 (Forma).

2.4. Reverse transcription (RT)-PCR

Total RNA was extracted using Trizol Reagent (Invitrogen) according to the manufacturer's instruction. RNA pellets were dissolved in diethylpyrocarbonate-treated water. The yield of RNA was quantified by spectroscopy at 260 nm. To synthesize first strand cDNA, 3 μg total RNA was incubated at 70 °C for 5 min with 0.5 μg of random hexamer and deionized water. The reverse transcription reaction was performed using 40 units of M-MLV reverse transcriptase (Promega) in 5× reaction buffer, RNase inhibitor at 1 unit/μl, and 2.5 mM dNTP mixtures at 37 °C for 60 min. The reaction was terminated by heating at 70 °C for 10 min, followed by cooling at 4 °C. The resulting cDNA samples were amplified at 94 °C for 5 min, 23 cycles of 94 °C for 45 s, 55 °C for 45 s and 72 °C for 45 s using Mastercycler gradient (Eppendorf). The primers used were: ER sense primer, 5′-CATAACGACTATATGTGTCCAGCC-3′; antisense primer, 5′-AACCGAGATGATGTAGCCAGCAGC-3′; β-actin sense primer, 5′-CCTGACCCTGAAGTACCCCA-3′, β-actin antisense primer, 5′-CGTCATGCAGCTCATAGCTC-3′. The expected size of amplicons for ERα and β-actin are 610 and 580 bp.

2.5. Transient transfection and luciferase assay

Cells were transiently transfected into the cell by calcium phosphate-DNA coprecipitation method. Luciferase activity was determined 24 h after drug treatments or exposing to hypoxia with an AutoLumat LB9507 luminometer using the luciferase assay system (Promega) and expressed as relative light units. The mean and standard deviation of duplicate samples are shown for representative experiments.

All transfection experiments were repeated three or more times with similar results.

2.6. Immunoblot analysis

Protein was isolated by using protein isolation buffer (containing 150 mM NaCl, 50 mM Tris–HCl, 5 mM EDTA, 1% Non-idet P-40, 0.5% deoxycholate, 1% SDS) with protease inhibitor cocktail (Sigma), 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% non-fat dry milk in Tris-buffered saline/0.05% Tween (TBST), and incubated with anti-rabbit polyclonal antibody to ERα (Santa Cruz Biotechnology) or to HIF-1α (BD Transduction Laboratories) or anti-mouse monoclonal antibody to β-actin (Sigma). After washing with TBST, blots were incubated with goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody and visualized with enhanced chemiluminescence kits (Amersham).

2.7. Immunoprecipitation

Two hundred micrograms (1 μg/μl) of the cell lysates were mixed with 1 μg of antibody (in 50 μl) and incubated overnight at 4 °C with constant rotation. To recover immunoprecipitated complexes, 150 μl of Protein A-sepharose (Upstate), 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 bound proteins were eluted by incubation in 5× SDS loading buffer for 5 min by boiling. The eluted proteins were analyzed by immunoblot analysis.

3. Results

3.1. Hypoxia regulates ER and estrogen-responsive luciferase genes

We and others have reported that hypoxia induces the proteasome-dependent downregulation of ER expression [16,20]. However, as in Fig. 1A, hypoxia represses steady-state levels of ER mRNA as well. This prompted us to investigate the effect of hypoxia on ligand-independent transcriptional activation of the ER. An ERE-responsive reporter plasmid was transiently transfected into MCF-7 cells. The transfected cells were treated as indicated and the cell lysates were assayed for luciferase activity. Hypoxia *per se* increased ERE-mediated transcription 2.3-fold after 24 h of treatment

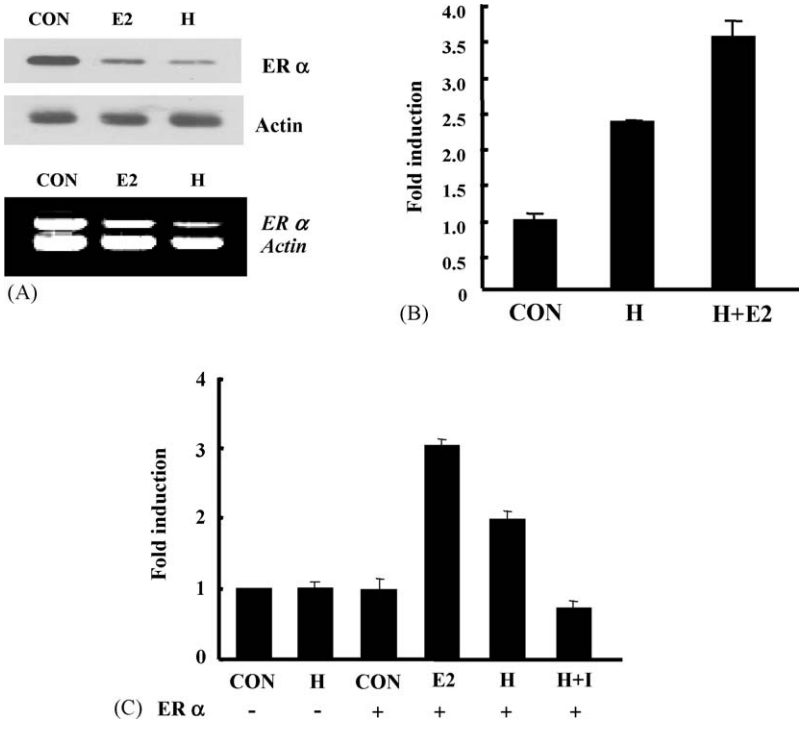

Fig. 1. Effects of hypoxia on ER and ER-mediated transcription activation. (A) MCF-7 cells were exposing to E2 or hypoxia (H) for 24 h. Total protein extracts were resolved by SDS-PAGE and immunoblotted using an anti-ERα or an anti-β-actin antibody (upper panel). Total RNA was analyzed for ERα and β-actin mRNA expression by RT-PCR assays (lower panel). (B) MCF-7 cells were transfected with ERE-Luc reporter construct and then the cells were untreated or treated with 10 nM E2 and incubated for 24 h under normoxic or hypoxic (H) conditions. Untreated groups served as a control (CON). (C) HEK293 cells were transfected with ERE-Luc reporter construct with or without expression vector for ERα. After transfection, cells were untreated or treated with 10 nM E2 or 1 μM ICI and incubated for 24 h under normoxic or hypoxic (H) conditions. After the incubation, the cells were lysed and luciferase expression was determined. Data are expressed as mean ± S.E.M. Each experiment was repeated at least three times.

(Fig. 1B). Co-treatment with 17-β-estradiol (E2) under conditions of hypoxia synergistically activated ERE-mediated transcription (Fig. 1B). Two distinct isoforms of ER, ERα and ERβ, have been identified. They are encoded by distinct genes located on different chromosomes. Studies have shown that ERα and ERβ have different transcriptional activities depending on the ligand, cell type, and promoter contexts [24]. Since MCF-7 cells express both ERα and ERβ proteins, it is difficult to separate the activities associated with each isoform. To focus our study on the effects of hypoxia on ERα-mediated transcription, we have used ER-negative HEK 293 cells transiently transfected with the ERα and ERE-luciferase genes. As shown in Fig. 1C, hypoxia increased, by approximately two-fold, ERE-driven luciferase expression in HEK 293 cells. No response was observed in the absence of ERα expression. To further confirm that the effects were ERα mediated, we co-treated the cells with the anti-estrogen ICI 182,780 (ICI), at a concentration that was sufficient to saturate almost all the ERs in the cells [25]. Transcriptional activation of the reporter plasmid by hypoxia was blocked by the addition of ICI, which indicates that the observed hypoxia-induced reporter gene activation was through ERα.

3.2. Hypoxia-induced ERα activation is mediated by HIF-1α

We have previously reported that both the GFP-tagged and endogenous HIF-1α interacts with ERα and that exogenously expressed ERα is able to precipitate GFP-tagged HIF-1α both under hypoxia and normoxia conditions (Fig. 2A) [20]. To specify the domain of ERα that interacts with HIF-1α and to further confirm the interaction between ERα and HIF-1α, we carried out mammalian two-hybrid assays using truncated ER fragments that were fused to the GAL4 DBD domain, in conjunction with the GAL4-Luc construct. The empty pVP16 vector served as a control. The transfection of HEK 293 cells with the HIF-1α N-terminus fused to the VP16 transactivation domain (H/V), GAL-ER_{DEF} or GAL-ER_{AB} and GAL4-Luc increased transactivation, as compared to cells that were transfected with VP16 and GAL-ER, which indicates strong interactions between HIF-1α and ER (Fig. 2B). Both the N-terminal and C-terminal regions of ERα interacted with HIF-1α. As a negative control, we have analyzed the interaction between DNA binding domain of the yeast GAL protein and HIF-1α. The transfection of HEK 293 cells with GAL (just containing DNA binding domain of the yeast GAL protein), GAL4-Luc, and H/V increased transactivation ~1.35-fold compared with cells transfected with GAL, GAL4-Luc and VP16, which indicates that the interactions between HIF-1 and DNA binding domain of GAL is insignificant (Fig. 2B). These results led us to investigate the role of HIF-1α in hypoxia-induced ERα transcriptional activation. Unfortunately, the instability of HIF-1α under normoxia conditions is an obstacle to studying this hypothesis. To circumvent this problem, we used GFP-tagged full length HIF-1α, as GFP fusion increases HIF-1α stability under normoxia conditions [26]. The function of the fusion protein that contained GFP and the full-length HIF-1α was maintained as examined by hypoxia-responsive elements driven reporter gene activation (data not shown). GFP-HIF-1α activated ERα-mediated transcription, as shown in Fig. 3A. In addition, ERα-mediated transactivation under hypoxia was dependent on the increased expression of GFP-HIF-1α (Fig. 3B). These data indicate that the hypoxic induction of ERα functional activation is mediated by HIF-1α.

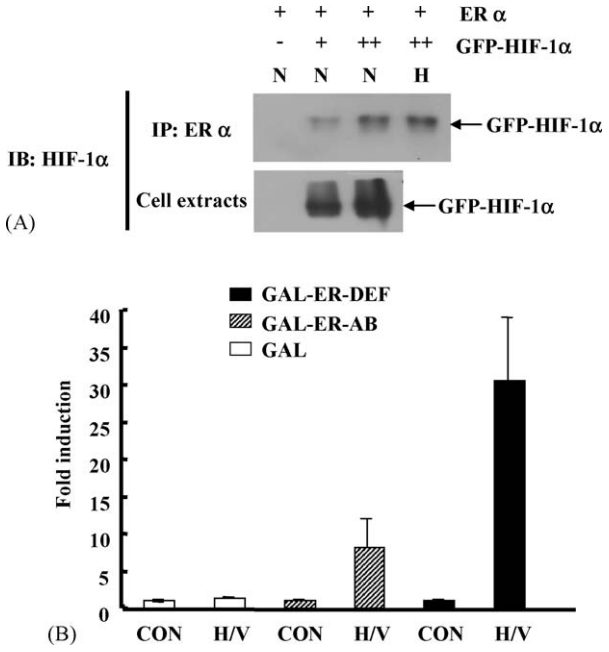

Fig. 2. Analyses of interaction between ERα and HIF-1α. (A) HEK 293 cells were transfected with 4 μg (+) or 8 μg (++) of expression vector for GFP-HIF-1α and/or 4 μg of expression vector for ERα as indicated, and then the cells were incubated for 22 h under normoxic (N) or hypoxic (H) conditions. Cell lysates were immunoprecipitated (IP) with anti-ERα antibody and precipitated proteins (upper panel) and total extracts (lower panel) were analyzed by immunoblots (IB) with anti-HIF-1α antibody. (B) HEK 293 cells were transiently transfected with GAL4-Luc reporter construct and the expression vector for GAL, GAL-$ER_{AB,}$ or GAL-ER_{DEF} fusion protein with or without the expression vector for HIF-1α/VP16 (H/V), and cells were incubated for 24 h. After the incubation, cells were lysed and luciferase expression was determined. Data are expressed as mean ± S.E.M. All the experiments were repeated at least two times.

4. Discussion

The growth of ER-positive breast cancer cells in vitro is dependent on agonist-occupied ER. In consequence, a clear understanding of the function and regulation of the ER is a prerequisite for optimal endocrine therapy for breast cancer [27]. Cellular hypoxia is a crucial stimulus in many pathological conditions, such as tumor growth. It has been suggested that hypoxia may be the key to the development of acquired hormone resistance in breast cancer by modulating ER expression levels [16,19,28]. However, the effects of hypoxia on ER transcriptional activation have not been

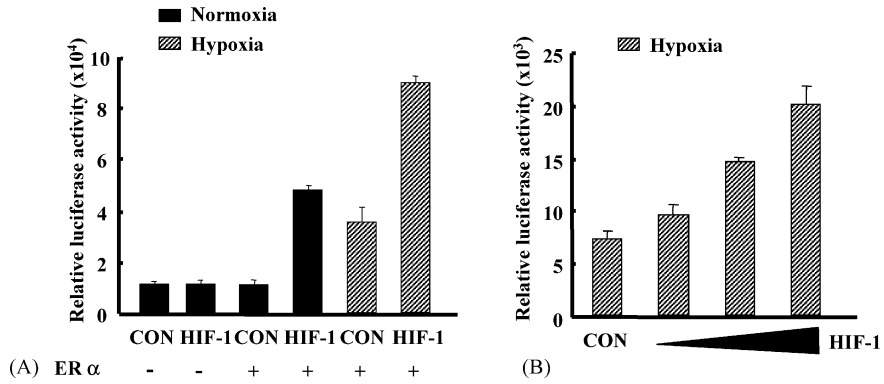

Fig. 3. Effects of HIF-1α expression on ERα transactivation under normoxic or hypoxic conditions. HEK 293 cells were transfected with ERE-Luc reporter construct (200 ng), with or without expression vector for ERα (200 ng), and with or without GFP-HIF-1α as indicated in the figure (A) or increasing concentration (100–400 ng) of GFP-HIF-1α (B). After transfection, cells were incubated for 24 h under normoxic (closed boxes) or hypoxic (hatched boxes) conditions. After this incubation, cells were lysed and luciferase expression was determined. Data are expressed as mean ± S.E.M. Each experiment was repeated at least two times.

clearly determined, despite its great importance in predicting the behavior of ER in the tumor environment. In this study, we have studied the modulation of ERα function as a transcription factor under hypoxia. We demonstrate that hypoxia activates ERα through HIF-1α, that ERα transactivation is enhanced by increased expression of the GFP-fused, full-length HIF-1α.

HIF-1α, AhR and their partner protein ARNT are members of the bHLH-PAS family [12]. This family shares considerable sequence homology with the p160 family of co-activators [4]. Recent studies have shown that AhR and ARNT modulate ER-dependent transcription by protein-protein association [4,7]. Our data show that both the N-terminus and C-terminus of ER interact with the bHLH-PAS domain of HIF-1α. This is in agreement with the finding that the bHLH-PAS domain of ARNT interacts with the DEF domain of ER, as shown in mammalian two hybrid assays [4]. Although they did not examine the interaction with the A/B domain of ER, deletion of the A/B domain reduced ER function. This indicates that both the A/B and DEF regions of the ERα are required for full activity [4]. However, the bHLH-PAS domain of AhR interacted directly with small regions of the A/B domain of ER that were mapped by in vitro glutathione *S*-transferase pull-down assays, and it is concluded that this interaction is required for AhR-induced activation of the ER function [7]. Additional detailed studies are required for the precise mapping of interaction region of ERα with HIF-1α.

We have recently reported that hypoxic induction of ER downregulation is mediated by HIF-1α, as examined using the HIF-1α/VP16 hybrid [20]. The Brown group has recently suggested that co-activator AIB1, which is a member of the p160 co-activator family and is encoded by a gene that is frequently amplified in breast cancers, is a dual-function protein in regulating ERα signaling [29]. AIB1 acts as a co-activator, while inducing ER proteasomal degradation. Accumulated studies have shown that dioxin-occupied AhR downregulates ER and activates ER [7,30]. Although further experiments are needed to demonstrate the precise role of HIF-1α in regulating ER, our data suggest that HIF-1α is likely to be another example of a dual-function ER-modulating protein. This does not necessarily support the notion that ER-mediated transactivation is coupled to ER protein downregulation. The possibility remains that the two functions are completely independent, and rely upon the nature of the recruited factors.

Our findings suggest that hypoxia can cause ERα activation, even in the absence of hormone and possibly through HIF-1α, which is associated with ER action in breast tumors. Since physiological stimuli other than hypoxia, such as insulin-like growth factor 1 (IGF-1) and activation of the muscarinic acetylcholine receptor, can also induce HIF-1α, this may be another signaling pathway for IGF-1-induced ER activation. Further studies are required to delineate the mechanism by which HIF-1α induces ERα activation, to determine the nature and significance of the interaction between ERα and HIF-1α, and to characterize the modulation of ER activity and degradation.

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