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Full length article

Hypoxia induced phosphorylation of estrogen receptor at serine 118 in the absence of ligand

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ABSTRACT

The estrogen receptor (ER) plays an important role in breast cancer development and progression. Hypoxia modulates the level of ERα expression and induces ligand-independent transcriptional activation of ERα, which is closely related with the biology of breast carcinomas. Since phosphorylation itself affects the transcriptional activity and stabilization of ERα, we examined changes in ERα phosphorylation under hypoxic conditions. Hypoxia induced phosphorylation of ERα at serine residue 118 (S118) in the absence of estrogen through the mitogen-activated protein kinase (MAPK)/ERK1/2 pathway. Cell proliferation was significantly decreased under normoxia or hypoxia when ERα harboring the S118A mutation was overexpressed. Our previous studies showed that ER degradation is the most prominent phenomenon under hypoxia. E2-induced ER protein downregulation is dependent on phosphorylation of S118. However, hypoxia-induced ERα degradation did not involve S118 phosphorylation. Our study implies the existence of a differential mechanism between E2 and hypoxia-mediated ERα protein degradation. Understanding the mechanistic behavior of ER under hypoxia will likely facilitate understanding of endocrine therapy resistance and development of treatment strategies for breast cancer.

1. Introduction

The estrogen receptor (ER) is an estrogen-activated transcription factor. Upon binding estrogen, ER undergoes conformational changes and post-translational modifications and regulates the transcription of target genes. Activation of ERα is initiated by ligand binding, but the function of ERα is also regulated by posttranslational modifications, most significantly by phosphorylation and ubiquitination. Even in the absence of estrogen, ER activity can be modulated by phosphorylation [1]. Phosphorylation of ERα affects various functions, including chromatin interaction, coregulator recruitment, and gene expression, and impacts breast tumor growth/morphology and the response of breast cancer patients to endocrine therapy. Studies have indicated that phosphorylation of ERα at serine residues 104, 106, 118, and 167 and tyrosine residue 537 by cellular kinases and the recruitment of co-activators serve as mechanisms for ligand-independent activation [2]. These serine residues, which are mostly clustered within the N-terminal AF-1 region, are phosphorylated by many kinases, including mitogen-activated protein kinase (MAPK), pp90rsk1, phosphoinositide-3-kinase (PI3 K)/AKT, and glycogen synthase kinase 3 (GSK-3). S167 is phosphorylated by pp90rsk and PI3 K/AKT [3]. Y537, within the C-terminal region, is phosphorylated by Src kinase. S118, the most well-characterized phosphorylation site of ERα, is phosphorylated by MAPK in a ligand-independent manner in vitro [4]. Phosphorylation of ERα at S118 induces steroid receptor coactivator 3 binding and ERα hypersensitivity to E2. Continuous activation of the MAPK pathway increases ERα S118 phosphorylation, bypassing the ligand-dependent ERα signaling pathway, thereby rendering tumors hormone-independent. S118 phosphorylation is a predictive marker for the response to tamoxifen, and tamoxifen inhibition of ERα-mediated tumor growth depends on S118 phosphorylation [5]. Investigation of the phosphorylated forms of ERα in human breast tumors revealed detectable levels of phosphorylated ERα in many breast tumor biopsy samples. The ERα phosphorylation score, determined by seven phosphorylated ERα epitopes, was significantly associated with overall breast cancer survival and relapse-free survival on multivariate analysis [6].

Hypoxia, defined as a loss of oxygen in tissues, is widespread in solid tumors because of the lack of sufficient vascularization for oxygen delivery [7]. In tumors, the mean oxygen tension is approximately 1.5% versus 7% in normal tissues [8]. The hypoxic environment initiates distinct signaling pathways, which allow for cell survival and adaptation to inadequate oxygen levels. The heterodimeric transcription factor hypoxia-inducible factor-1 (HIF-1) is the master regulator of hypoxic responses. It consists of an oxygen-sensitive α subunit coupled to a constitutively expressed β subunit. Under hypoxic conditions, stabilized

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HIF-1α translocates to the nucleus, dimerizes with HIF-1β, and regulates transcription of HIF-1-responsive genes by binding to the hormone response element in the 5′ flanking region of target genes [9–12]. Several reports have indicated a correlation between hypoxia and malignant breast cancer [13,14]. HIF-1α protein levels are lower in normal human breast epithelium compared with that of breast cancer [15]. HIF-1α overexpression is associated with increased proliferation in breast cancer. ER-positive and ER-negative tumors showed differential expression of biomarkers suggesting an interaction between HIF-1α and ER [16]. Clinical studies indicated that expression of the HIF-1α is associated with a poor survival to antiestrogen treatment in ER-positive breast cancer [17,18]. Thus, it is thought that HIF-1α expression influences the molecular pathogenesis of breast cancer.

We and others have shown that hypoxia activates and destabilizes ER [19,20]. Hypoxia induces the degradation of ER within 6–12 h via a proteasome-dependent pathway, which is dependent on HIF-1α expression and the physical interaction between ERα and HIF-1α. Estrogen activates HIF-1α and induces the recruitment of both ERα and HIF-1α to the promoter of the vascular endothelial growth factor gene; this was shown to be dependent on the PI3 K/AKT pathway in rat uterus using chromatin immunoprecipitation assays [21]. However, the molecular pathways involved in the crosstalk between ERα and HIF-1α remain largely unknown. In this study, we examined the influence of phosphorylation of the ERα S118 residue on hypoxia-mediated transcriptional activation, ERα protein degradation, and cell proliferation and migration. Our results demonstrated that ERα is phosphorylated at S118 by hypoxia in the absence of a ligand, thereby affecting cell proliferation, but S118 phosphorylation does not affect hypoxia-induced ERα-mediated transcriptional activity or protein degradation.

2. Materials and methods

2.1. Reagents

17-β-estradiol (E2), PD98059 were purchased from Sigma (St. Louis, MO). PD98059 was dissolved in dimethylsulfoxide (DMSO) and E2 in 100% ethanol. All the compounds were added to the culture media 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. The pSG5-full length ERα was from Dr. Pierre Chambon. ERα S118A phosphorylation mutant expression plasmid was kindly provided by Dr. Guan Chen (Medical College of Wisconsin, Wisnonsin, USA) [22].

2.3. Cell culture and hypoxic condition

MCF-7 and MDAMB-231 cells were maintained in phenol red-free RPMI 1640 supplemented with 10% fetal bovine serum (FBS; WelGENE, Korea). HEK 293 cells were maintained in phenol red-free Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% FBS. 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 RPMI 1640 or DMEM/5% charcoal-dextran stripped FBS (CD-FBS) for 2 days to eliminate any estrogenic source before treatment. 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. Transient transfection and luciferase assay

Cells were transiently transfected into the cell by using the polyehylenimine (Polyscience, Warrington, PA, USA). Luciferase activity was determined 24 h after drug treatments or exposing to hypoxia with an AutoLumat LB9507 luminometer using the luciferase assay system (Promega, Madison, WI, USA) and expressed as relative light units. The mean and standard deviation of triplicate samples are shown for representative experiments. All transfection experiments were repeated three or more times with similar results.

2.5. Western blot analysis

Protein was isolated as described previously. After SDS-PAGE, proteins were transferred to a polyvinylidene difluoride membrane, blocked with 5% nonfat dry milk in Tris-buffered saline/0.05% Tween (TBST). The membranes were incubated for 2 h at room temperature or overnight at 4 °C with primary antibodies. After washing with TBST, blots were incubated with goat anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibody and visualized with enhanced chemiluminescence detection kits (GE Healthcare, Piscataway, NJ). Anti-ERα (SC-543), anti-phospho-ERK1/2 (SC-16982), anti-ERK1/2 (SC-135900), anti-Lamin B (SC-6216) from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-phospho-ERα Ser118 from Upstate cell signaling solutions (07-487, 07-481, Upstate, NY, USA) and anti-HIF-1α (610959) from BD Biosciences and anti-β-actin (A5316) from Sigma were used.

2.6. Reverse transcription (RT)-Polymerase chain reaction (PCR)

Total RNA was extracted using Trizol Reagent according to the manufacturer's instruction. 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 (up to 11 μl). The reverse transcription reaction was performed using 40 units of M-MLV reverse transcriptase (Promega, Madison, WI, USA) in 5 × reaction buffer, RNase inhibitor at 1 unit/μl, and 1 mM dNTP mixtures at 37 °C for 60 min. 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′; ESR1 sense primer, 5′-CTACTGTGCAGTGTGCAATGACTA-3′; ESR1 anti-sense primer, 5′-ATGTCCTTGAATACTCTCTTGAAG-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.

2.7. Nuclear extract preparation

Nuclear proteins were prepared by using Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to the manufacturer's protocol. Cells were washed with 1 ml ice-cold PBS/phosphatase inhibitors, lysed in 500 μl hypotonic buffer and then centrifuged at 14,000*g* for 30 s at 4 °C. Pellets were resuspended in complete lysis buffer, and centrifuged at 14,000*g* for 10 min at 4 °C, and supernatants containing nuclear fraction were recovered.

2.8. Statistical analysis

All *in vitro* data were analyzed and expressed as means and standard deviations (SD). The two-tailed, unpaired Student *t*-test was applied using SPSS software (version 23.0; IBM, Armonk, NY, USA).

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