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Hypoxia enhances transcriptional activity of androgen receptor through hypoxia-inducible factor-1α in a low androgen environment

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ABSTRACT

The androgen receptor (AR) acts as a ligand-dependent transcriptional factor controlling development or progression of prostate cancer. Androgen ablation by castration is an effective therapy for prostate cancer, whereas eventually most of the tumors convert from a hormone-sensitive to a hormone-refractory disease state and grow even in a low androgen environment (e.g., 0.1 nM 5α-dihydrotestosterone (DHT)) like the castration-resistant stage. Androgen ablation results in hypoxia, and solid tumors possess hypoxic environments. Hypoxia-inducible factor (HIF)-1, which is composed of HIF-1α and HIF-1β/ARNT subunits, functions as a master transcription factor for hypoxia-inducible genes. Here, we report that hypoxia enhances AR transactivation in the presence of 0.05 and 0.1 nM DHT in LNCaP prostate cancer cells. siRNA-mediated knockdown of HIF-1α inhibited hypoxia-enhanced AR transactivation. Its inhibition by HIF-1α siRNA was canceled by expression of a siRNA-resistant form of HIF-1α. HIF-1α siRNA repressed hypoxia-stimulated expression of the androgen-responsive *NKX3.1* gene in the presence of 0.1 nM DHT, but not in the absence of DHT. In hypoxia, HIF-1α siRNA-repressed AR transactivation was restored in mutants in which HIF-1α lacked DNA-binding activity. Furthermore, a dominant negative form of HIF-1α canceled hypoxia-enhanced AR transactivation, and HIF-1β/ARNT siRNAs had no influence on hypoxia-enhanced AR transactivation. These results indicate that hypoxia leads to HIF-1α-mediated AR transactivation independent of HIF-1 activity and that HIF-1β/ARNT is not necessarily required for the transactivation.

1. Introduction

Androgens are steroid hormones that are required for not only the development and maintenance of normal prostate, but also the development and progression of prostate cancer [1,2]. Androgens, principally testosterone and its more potent metabolite, 5α-dihydrotestosterone (DHT), bind to the androgen receptor (AR) in the cytoplasm, leading to a conformational change of AR to an active form. The ligand-bound AR translocates into the nucleus and binds to androgen response elements (AREs) on the target gene promoters, resulting in increased expression of the target genes [3]. The transcriptional activity of AR requires the recruitment of coactivators.

The survival and proliferation of prostate cancer cells are initially suppressed in response to androgen ablation by medical or surgical castration, which decreases serum androgen levels to about 0.1 nM DHT [4]. However, a few years after castration, most of the tumors become androgen-refractory and grow despite low serum androgen levels. Thus, almost all patients eventually progress to fatal castration-resistant prostate cancer (CRPC) [5–7]. In CRPC, AR signaling, which has been inactivated by androgen ablation, can be reactivated in several ways, including (1) increased AR expression, (2) AR gene mutations leading to enhancement of the ligand response or induction of the ligand-independent response, (3) alterations in AR coactivators/corepressors resulting in enhanced AR transactivation, and (4) activation of AR function due to crosstalk with other signaling pathways [8].

Androgen ablation by castration results in hypoxia due to insufficient blood flow in the prostate tissue [9,10]. Hypoxia is a key factor in tumorigenesis because solid tumors possess unique microenvironments that are insufficiently supplied with oxygen [11]. The transcriptional activation of hypoxia-inducible genes as an intracellular adaptive response to hypoxia is mediated by the stabilization and activation of hypoxia-inducible factor-1 (HIF-

Abbreviations: AR, androgen receptor; ARE, androgen response element; CRPC, castration-resistant prostate cancer; C-TAD, C-terminal transactivation domain; DHT, 5α-dihydrotestosterone; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HIF-1, hypoxia-inducible factor-1; HRE, hypoxia-responsive element; N-TAD, N-terminal transactivation domain; PSA, prostate-specific antigen.

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1) [12]. HIF-1 is a heterodimeric protein composed of HIF-1α and HIF-1β/ARNT subunits. The HIF-1α subunit contains a basic helix-loop-helix, a PER-ARNT-SIM domain, and two transactivation domains (N-TAD and C-TAD, located in the N-terminal and C-terminal regions, respectively). In normoxia, the HIF-1α subunit is hydroxylated on two conserved proline residues, and the resultant proline-hydroxylated HIF-1α undergoes ubiquitination, followed by proteasomal degradation. In hypoxia, however, the HIF-1α subunit is stably expressed due to avoidance of proline hydroxylation [13]. On the other hand, the HIF-1β/ARNT subunit is constitutively expressed. Thus, in hypoxia, the stabilized HIF-1 heterodimer binds to hypoxia-responsive elements (HREs) in the target hypoxia-inducible genes, resulting in transcriptional activation of their genes [14].

Relapsed hormone-refractory prostate cancer cells highly express nuclear HIF-1α [15], suggesting that the castration-resistant tumor cells exist in hypoxia. However, it remains unclear whether hypoxia or HIF-1α is involved in AR signaling in CRPC cells. In the present study, we report that at a low DHT concentration mimicking the castration-resistant stage, hypoxia enhances the transcriptional activity of AR and elevates the expression of the androgen-responsive *NKX3.1* gene in LNCaP prostate cancer cells. Furthermore, we demonstrate that HIF-1α, but not HIF-1β/ARNT, is required for hypoxia-enhanced AR transactivation.

2. Materials and methods

2.1. Cell culture

LNCaP (AR-positive human prostate cancer) cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin as described previously [16] unless otherwise indicated. For hypoxic exposure, cells were placed in a MCO-5M multi-gas incubator (Sanyo Electric Co., Ltd., Japan) flushed with 1% O_2, 5% CO_2 and 94% N_2 at 37 °C at 100% humidity.

2.2. siRNA

Double-stranded siRNA for human HIF-1α was chemically synthesized (Dharmacon, Chicago, IL). Sequences of the HIF-1α siRNA were designed as follows: sense strand 5′-CUGAUGACCAGCAACUU GAdTdT-3′ and antisense strand 5′-UCAAGUUGCUGGUCAUCA GdTdT-3′ [17]. Human HIF-1β/ARNT siRNAs and control siRNA were purchased from Dharmacon (product number, ARNT#1: J-007207-06-0005 and ARNT#2: J-007207-07-0005; and control: D-001140-01-20). LNCaP cells were transiently transfected with control, HIF-1α, or HIF-1β/ARNT siRNA duplexes at 20 nM for 6 h using DharmaFECT 4 (Dharmacon) according to the manufactures' instructions.

2.3. Plasmids

The androgen-responsive reporter plasmid (pARE-Luc) and the hypoxia-responsive reporter plasmid (pEpo-HRE-Luc) were constructed. The pARE-Luc is a luciferase reporter driven by a minimal promoter with two AREs and no putative HREs, and the pEpo-HRE-Luc is a luciferase reporter driven by a minimal promoter with three HREs mimicking HRE in the erythropoietin gene. In addition to pEpo-HRE-Luc with artificial HREs, a reporter plasmid to estimate HIF-1α activity was constructed by introducing the promoter region (nucleotide sequence: −1091 to +25) of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene with HREs [18] into pGL3-basic vector (Promega Corp., Madison, WI, USA), termed pGAPDH-HRE-Luc. Human AR mammalian expression vector (pcDNA3.1-AR) was described previously [19]. The cDNAs encoding human HIF-1α (GenBank accession no. NM_001530) and HIF-1α^{516} [20], which is a dominant negative form of HIF-1α, were amplified by PCR. The HIF-1αDM cDNA encoding mutant HIF-1α substituting Ala for Pro at positions 402 and 564 was amplified by PCR using specific primers, and the N-terminal HA-tagged HIF-1αDM and HIF-1α^{516} expression vectors (pcDNA3.1-HA-HIF-1αDM and pcDNA3.1-HA-HIF-1α^{516}) were constructed. A HIF-1α mutant cDNA encoding a siRNA-resistant form of HIF-1α, designed HIF-1α(mut), was synthesized by site-directed mutagenesis of pcDNA3.1-HA-HIF-1αDM using mutation primers, 5′-CaGAcGAtCAaCAgCTgGA-3′ and 5′-TCcAGcTGtTGaTCgTCtG-3′ (lower-case letters indicate mutation sites), followed by construction of pcDNA3.1-HA-HIF-1α(mut). The HIF-1α(A26E) and HIF-1α(R30A) cDNAs encoding HIF-1α mutants substituting Glu for Ala at position 26 and Ala for Arg at position 30, respectively, were amplified by PCR using pcDNA3.1-HA-HIF-1α(mut) as a template, and the resultant plasmid vectors were termed pcDNA3.1-HA-HIF-1α(A26E) and pcDNA3.1-HA-HIF-1α(R30A), respectively.

2.4. Reporter assay and Western blot analysis

For reporter assay, LNCaP cells were grown on 24-well plates in steroid-free RPMI 1640 medium supplemented 10% fetal bovine serum and transiently transfected using HilyMax reagent (Dojindo Laboratories, Kumamoto, Japan). Transfection efficiency was normalized using pRL-SV40 (*Renilla* luciferase expression vector, Promega Corp., Madison, WI, USA). For detection of endogenous HIF-1α, ARNT, and α-tubulin, LNCaP cells were lysed in 20 mM Hepes-NaOH, pH 7.5, containing 150 mM NaCl, 0.5% NP-40, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 10 μg/ml leupeptin, and 1 μg/ml aprotinin. Cell lysates were analyzed by Western blotting with mouse monoclonal anti-HIF-1α (1/2000, Clone: mgc3, Affinity BioReagents, Golden, CO, USA), mouse monoclonal anti-ARNT (1/3000, Clone: 29, BD Transduction Laboratories, San Diego, CA, USA), and mouse monoclonal anti-α-tubulin (1/5000, Clone: DM 1A, Sigma, St. Louis, MO, USA) antibodies.

2.5. Immunofluorescent microscopy

Immunofluorescent microscopy was performed as described previously [19]. LNCaP cells were cultured in steroid-free RPMI 1640 medium supplemented 10% fetal bovine serum on round coverglasses on 12-well plates. Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, and 1.47 mM KH_2PO_4, pH 7.4), permeated, and incubated with mouse monoclonal anti-HIF-1α (1/500) or mouse monoclonal anti-ARNT (1/1000). Cells were further incubated with Alexa Fluor 488-conjugated secondary anti-mouse IgG (1/5000) (Molecular Probes, Eugene, OR, USA) and labeled with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, 1 μg/ml) to stain nuclear chromatin, followed by inspection using a fluorescence microscope (model BZ-9000; Keyence, Osaka, Japan).

2.6. Semi-quantitative RT-PCR

Total RNAs were extracted from the LNCaP cells with Sepasol-RNA Super (Nacalai Tesque, Kyoto, Japan), and cDNAs were synthesized by reverse transcriptase. The gene expression of *NKX3.1*, *PSA*, *AR*, and *β-actin* was determined by PCR using the following primer pair: *NKX3.1* (forward primer 5′-CTGTTATACA CGGAGACCAGG-3′ and reverse primer 5′-GTACCTGTCGGCCCCT GAACG-3′), *PSA* (forward primer 5′-ACCCTCAGAAGGTGACCAAGT-3′ and reverse primer 5′-TGAAGCACACCATTACAGACA-3′), *AR* (forward primer 5′-CCTGGCTTCCGCAACTTACAC-3′ and reverse primer 5′-GGACTTGTGCATGCGGTACTCA-3′), *HIF-1α* (forward