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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

primer 5′-CATCCAAGAAGCCCTAACGTGT-3′ and reverse primer 5′-TTAACTTGATCCAAAGCTCTGAG-3′), and *β-actin* (forward primer 5′-TGGAGTCCTGTGGCATCCACGAAA-3′ and reverse primer 5′-TGTAACGCAACTAAGTCATAGTCCG-3′). The PCR profiles consisted of denaturation at 95 °C for 1 min, primer-annealing at 55 °C for 1 min, and primer extension at 72 °C for 30 s. The final primer extension was performed at 72 °C for 10 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel and photographed under UV light. The relative expression levels of mRNAs were calculated by determining the ratio of the amount of each mRNA to that of endogenous reference gene, *β-actin*.

2.7. Statistics

Data were logarithmically transformed to improve normality and to compensate for unequal variance and assessed by two-way analysis of variance (ANOVA) with Tukey's *post hoc* testing. Statistical analysis was performed using JMP statistical software version 8.0.1 (SAS Institute, Cary, NC, USA). Date are expressed as means ± SD, and differences were considered statistically significant at a p value of <0.05.

3. Results

3.1. Hypoxia enhances ligand-activated AR transactivation at a low level of DHT

To assess the effect of hypoxia on AR transactivation in prostate cancer cells, LNCaP cells were exposed to hypoxia at various concentrations of DHT. Hypoxia enhanced AR transactivation in the presence of 0.05 and 0.1 nM DHT, but not 0.2–10 nM, although DHT activated AR transactivation at any concentrations tested under both normoxic and hypoxic conditions (Fig. 1). The artificial ARE reporter plasmid used to estimate the transcriptional activity of AR has no putative HREs to respond to hypoxia, and in fact, hypoxia had no influence on AR transactivation in the absence of DHT. These results indicate that hypoxia enhances AR transactivation through ARE, but probably not through HRE, at a low DHT level (0.1 nM DHT) mimicking the castration-resistant stage.

3.2. HIF-1α is involved in hypoxia-enhanced AR transactivation at a low level of DHT

We determined whether HIF-1α is required for the hypoxia-induced transcriptional activity of AR. To study the effectiveness of

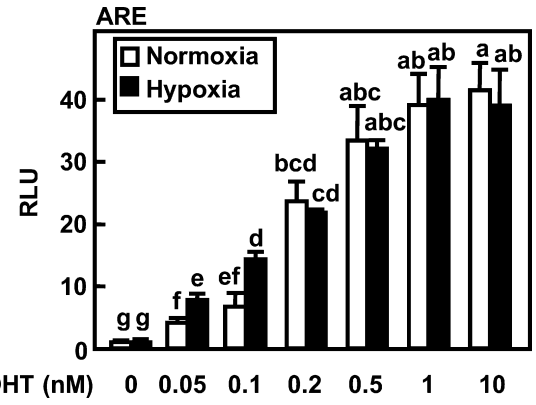

Fig. 1. AR transactivation in hypoxia at low levels of DHT. LNCaP cells were cultured for 48 h and transiently transfected with pcDNA3.1-AR, pARE-Luc, and pRL-SV40 for 24 h, followed by incubation with DTH at various concentrations in normoxia (21% O_2) or hypoxia (1% O_2) for an additional 9 h. Luciferase activities were determined, and data are expressed as means ± SD (n = 3). Statistically significant differences (p < 0.05) are indicated by different letters. The result is representative of three independent experiments.

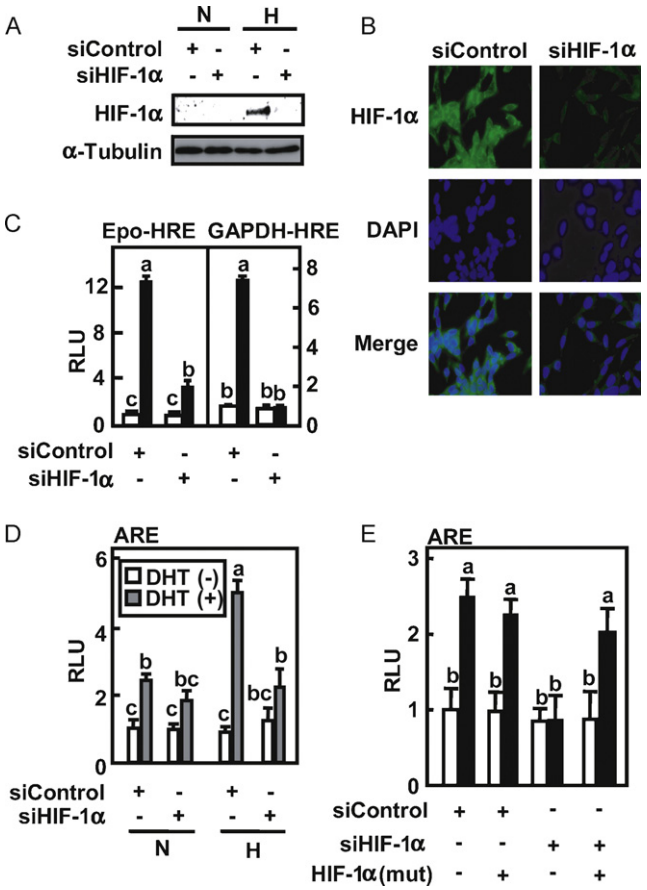

Fig. 2. Involvement of HIF-1α in hypoxia-enhanced AR transactivation. (A) LNCaP cells were treated with 20 nM HIF-1α siRNA (siHIF-1α) or control siRNA (siControl) for 6 h, followed by incubation for 42 h. Cells were incubated in normoxia (N) or hypoxia (H) for an additional 9 h. Cell lysates were prepared and analyzed by Western blot using anti-HIF-1α and anti-α-tubulin antibodies. The result is representative of three independent experiments. (B) After HIF-1α siRNA treatment, cells were cultured for 18 h, followed by exposure to hypoxia for an additional 9 h. Cell were fixed, permeated, and incubated with anti-HIF-1α IgG. Immunoreactive HIF-1α was visualized using Alexa Fluor 488-conjugated secondary anti-mouse IgG. The nuclear chromatin was stained with DAPI, and random fields were photographed. Quantification of cells stained by DAPI or immunoreacted with anti-HIF-1α antibodies was performed. Values are calculated from data of random fields, and results are representative of two independent experiments. (C) After HIF-1α siRNA treatment, cells were cultured for 18 h, followed by transfection with pEpo-HRE-Luc (left panel) or pGAPDH-HRE-Luc (right panel), and pRL-SV40 for 24 h. Cells were incubated in normoxia (white bars) or hypoxia (black bars) for an additional 9 h. (D) After siRNA treatment, cells were cultured for 18 h, transfected with pcDNA3.1-AR, pARE-Luc, and pRL-SV40 for 24 h. Cells were incubated in the presence and absence of 0.1 nM DHT for an additional 9 h in normoxia (N) or hypoxia (H). (E) After siRNA treatment, pcDNA3.1-HA-HIF-1α(mut) was co-transfected with pcDAN3.1-AR, pARE-Luc and pRL-SV40 for 24 h. Cells were incubated in the presence of 0.1 nM DHT for an additional 9 h in normoxia (white bars) or hypoxia (black bars). In this assay, the amount of DNA was kept constant by addition of pcDNA3.1-HA-Myc-His empty vector. (C–E) Luciferase activities were determined. Three independent experiments were performed, and data are expressed as means ± SD of a representative experiment (n = 3). Statistically significant differences (p < 0.05) are indicated by different letters.

the designed HIF-1α siRNA, LNCaP cells were transfected with HIF-1α-specific siRNA, followed by exposure to hypoxia. HIF-1α siRNA specifically knocked down the expression of hypoxia-induced HIF-1α protein, but not α-tubulin (Fig. 2A). In addition, to assess the efficiency of HIF-1α siRNA-meidated knockdown, immunofluorescence analysis was performed in siRNA-treated cells. HIF-1α was knocked down by HIF-1α siRNA in almost all cells, indicating high transfection efficiency of siRNA (>90%) (Fig. 2B). HIF-1α siRNA inhibited hypoxia-activated HIF-1 activity not only on the artificial HRE reporter (Epo-HRE), but also on the authentic HRE reporter (GAPDH-HRE) (Fig. 2C), indicating the specificity and

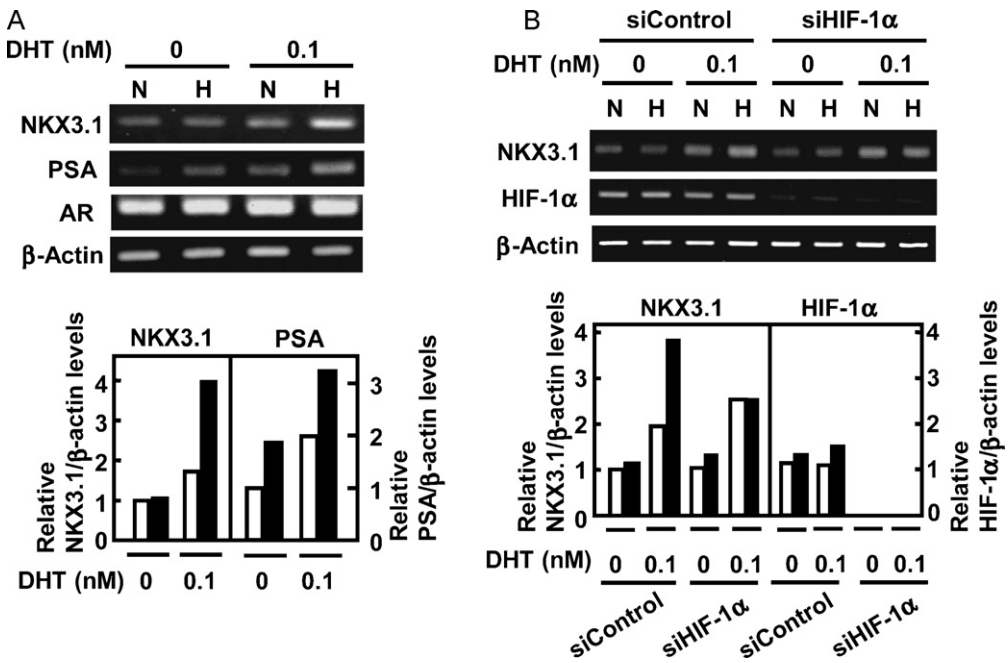

Fig. 3. HIF-1α-dependent expression of *NKX3.1* gene at a low level of DHT in hypoxia. (A) LNCaP cells were cultured for 72 h, followed by incubation in the presence and absence of 0.1 nM DHT in normoxia (N) or hypoxia (H) for an additional 9 h. (B) Cells were treated with 20 nM siHIF-1α or siControl, followed by incubation for an additional 42 h. Subsequently, cells were cultured in the presence and absence of 0.1 nM DHT in normoxia or hypoxia for an additional 9 h. Total RNAs were extracted, and the mRNA levels were semi-quantitatively measured by RT-PCR (upper panel). The abundance of *β-actin* mRNA was semi-quantitatively measured as an internal standard. In normoxia (white bars) or hypoxia (black bars), relative mRNA levels were calculated as a ratio for mRNA from cells in the absence of DHT in normoxia (lower panel). The result is representative of three independent experiments.

effectiveness of HIF-1α siRNA used here. When the effect of HIF-1α siRNA on hypoxia-enhanced AR transactivation was assessed, the siRNA-mediated knockdown of HIF-1α repressed hypoxia-enhanced AR transactivation in the presence of 0.1 nM DHT (Fig. 2D). Furthermore, to determine whether HIF-1α is involved in AR transactivation in hypoxia, HIF-1α was knocked down in LNCaP cells, and HIF-1α(mut), which is a HIF-1α siRNA-resistant and the O_2-insensitive form, was overexpressed, followed by exposure to hypoxia. HIF-1α(mut) had an enhancing effect on AR transactivation when hypoxia-activated AR transactivation was canceled by HIF-1α siRNA (Fig. 2E). In contrast, HIF-1α(mut) exhibited no additional effect on hypoxia-activated AR transactivation when cells were treated by control siRNA in hypoxia.

3.3. HIF-1α is involved in hypoxia-increased AR-responsive gene expression at a low level of DHT

To determine whether hypoxia at a low DHT level actually affects the expression of androgen-responsive genes such as the human prostate-specific antigen (*PSA*) [21] and *NKX3.1* [22], LNCaP cells were exposed to hypoxia in the presence and absence of 0.1 nM DHT. As shown in Fig. 3A, *NKX.3.1* gene expression was induced by hypoxia in the presence of 0.1 nM DHT, but not in the absence of DHT, whereas *PSA* gene expression was increased by hypoxia both in the presence and absence of 0.1 nM DHT. On the other hand, hypoxia had no influence on the expression of *β-actin* mRNA (a negative control) and *AR* mRNA. Subsequently, we assessed the effect of HIF-1α siRNA-mediated knockdown on the expression of *NKX3.1* gene. When LNCaP cells were transfected with HIF-1α siRNA in hypoxia, *HIF-1α* mRNA expression was repressed in the presence or absence of DHT in hypoxia (Fig. 3B). *NKX.3.1* mRNA expression was increased by hypoxia in the presence of DHT, whereas HIF-1α siRNA inhibited the hypoxia-increased *NKX.3.1* mRNA expression. In addition, when the inhibitory effects of HIF-1α siRNA on hypoxia-increased *NKX3.1* mRNA expression in the presence of 0.1 nM DHT were determined by real-time quantitative RT-PCR analysis, similar results were obtained (data not shown). These results indicate that HIF-1α is involved in hypoxia-enhanced AR-responsive gene expression in the presence of 0.1 nM DHT.

3.4. HIF-1α enhances AR transactivation independent of binding ability to HRE

Hypoxia enhanced AR transactivation through ARE, but probably not through HRE. To determine whether HIF-1α enhances AR transactivation without binding to HRE in hypoxia, HIF-1α(A26E) and HIF-1α(R30A), which lack binding ability to HRE [23], were constructed from HIF-1α(mut) as a template. Because HIF-1α exerts HIF-1 activity through binding to HRE, in HIF-1α(A26E) and HIF-1α(R30A) mutants, the inhibitory effect of HIF-1α siRNA on HIF-1 activity was not attenuated, although in a HIF-1α(mut) mutant (a positive control), the inhibitory effect was restored (Fig. 4A). In contrast, in HIF-1α(A26E) and HIF-1α(R30A) mutants, the HIF-1α siRNA-repressed AR transactivation in hypoxia was restored (Fig. 4B). These results indicate that HIF-1α enhances AR transactivation independent of binding ability to HRE.

3.5. A dominant negative form of HIF-1α inhibits hypoxia-enhanced AR transactivation

Because HIF-1α exerts HIF-1 activity through its N-TAD and C-TAD [14], HIF-$1\alpha^{516}$ lacking the C-terminal part of N-TAD and the C-TAD functions as a dominant negative form of HIF-1α [20]. In fact, HIF-$1\alpha^{516}$ inhibited hypoxia-activated HIF-1 activity (Fig. 5, left panel). To assess the effect of HIF-$1\alpha^{516}$ on hypoxia-enhanced AR transactivation, LNCaP cells were overexpressed HIF-$1\alpha^{516}$, followed by exposure to hypoxia in the presence of 0.1 nM DHT. Overexpression of HIF-$1\alpha^{516}$ repressed hypoxia-enhanced AR transactivation (Fig. 5, right panel).

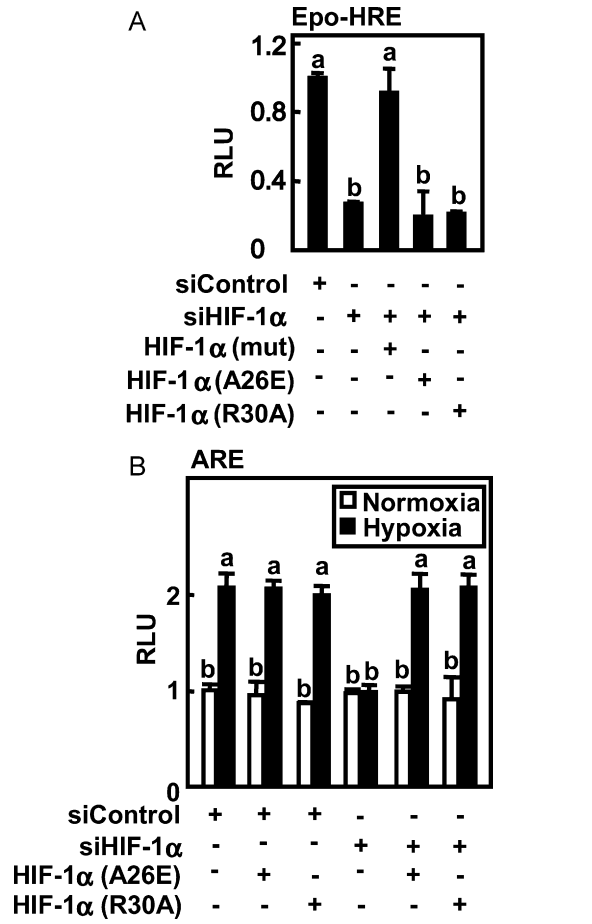

Fig. 4. HIF-1α-enhanced AR transactivation independent of binding to HRE. (A) LNCaP cells were cultured for 24 h and treated with 20 nM siHIF-1α or siControl for 6 h, followed by incubation for 18 h. The reporter plasmids (pEpo-HRE-Luc and pRL-SV40) were transfected into cells with pcDNA3.1-HA-HIF-1α(mut), pcDNA3.1-HA-HIF-1α(A26E), or pcDNA3.1-HA-HIF-1α(R30A) for 24 h. Cells were incubated in the presence of 0.1 nM DHT for an additional 9 h in hypoxia. (B) After HIF-1α siRNA treatment, cells were transfected with pcDNA3.1-AR, pARE-Luc, pRL-SV40, and pcDNA3.1-HA-HIF-1α(A26E) or pcDNA3.1-HA-HIF-1α(R30A) for 24 h. Cells were incubated in the presence of 0.1 nM DHT for an additional 9 h in normoxia or hypoxia. In this assay, the amount of DNA was kept constant by addition of pcDNA3.1-HA-Myc-His empty vector. Luciferase activities were determined. Three independent experiments were performed, and data are expressed as means ± SD of a representative experiment ($n = 3$). Statistically significant differences ($p < 0.05$) are indicated by different letters.

3.6. HIF-1β/ARNT is not necessarily required for hypoxia-enhanced AR transactivation

HIF-1α forms a heterodimer with HIF-1β/ARNT to exert HIF-1 activity in response to hypoxia [14]. To investigate the involvement of HIF-1β/ARNT in hypoxia-enhanced AR transactivation at a low level of DHT, LNCaP cells were transfected with either of two kinds of HIF-1β/ARNT siRNAs, followed by exposure to hypoxia. Either HIF-1β/ARNT siRNA inhibited the expression of HIF-1β/ARNT protein (Fig. 6A). Knockdown of HIF-1β/ARNT was observed in almost all cells, indicating high transfection efficiencies of siRNAs (>80%) (Fig. 6B). In addition, HIF-1β/ARNT siRNAs repressed hypoxia-induced HIF-1 activities on an artificial HRE reporter (Epo-HRE) and the authentic HRE reporter (GAPDH-HRE) (Fig. 6C). In contrast, siRNA-mediated knockdown of HIF-1β/ARNT had no influence on hypoxia-activated AR transactivation at 0.1 nM DHT (Fig. 6D). These results indicate that HIF-1β/ARNT is not required for hypoxia-enhanced AR transactivation at a low level of DHT.

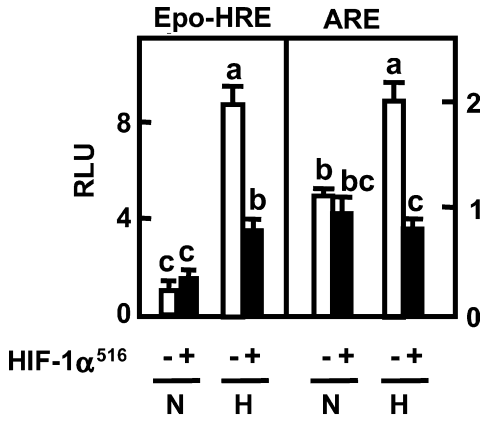

Fig. 5. Effect of a dominant negative form of HIF-1α on hypoxia-enhanced AR transactivation. LNCaP cells were transfected with pcDNA3.1-HA-HIF-1α^{516} and pRL-SV40 together with pEpo-HRE-Luc (left panel), or pcDNA3.1-AR and pARE-Luc (right panel) for 24 h. Cells were incubated in the presence of 0.1 nM DHT for an additional 9 h in normoxia or hypoxia. The amount of DNA was kept constant by addition of pcDNA3.1-HA-Myc-His empty vector. Luciferase activities were determined. Three independent experiments were carried out, and data are expressed as means ± SD of a representative experiment ($n = 3$). Statistically significant differences ($p < 0.05$) are indicated by different letters.

4. Discussion

In patients with prostate cancer, the mean total serum DHT concentrations before and after androgen deprivation therapy are 490 pg/ml (approx. 2 nM) and 38 pg/ml (approx. 0.1 nM), respectively [4]. Androgen ablation is an effective therapy for androgen-dependent prostate cancer. However, prostate cancer switches from a hormone-sensitive state to a hormone-refractory state, and consequently progresses as a CRPC even in a low (but not zero) androgen environment after androgen deprivation therapy [24]. Although the activated AR signaling pathway is involved in the progression to CRPC, the mechanisms of the progression to CRPC remain unclear. Androgen ablation by castration results in hypoxia [9,10], and castration-resistant cancer cells highly express nuclear HIF-1α, which is stably expressed in hypoxia [15]. Therefore, we focused on the AR signaling in hypoxia in CRPC. In the present study, we demonstrate that hypoxia enhances AR transactivation at a low level of DHT (0.1 nM) mimicking the castration-resistant stage. In contrast, hypoxia had no influence on AR transactivation in the presence of DHT (0.2–10 nM) or absence of DHT, consistent with the previous literature [17].

Semi-quantitative RT-PCR analyses revealed that hypoxia induced the expression of *PSA* mRNA in both the presence (0.1 nM) and absence of DHT and the expression of *NKX3.1* mRNA in the presence of 0.1 nM DHT, but not in the absence of DHT. A previous study showed that the expressions of *PSA* and *NKX3.1* mRNAs in LNCaP cells are increased by 10 nM DHT and that *PSA* mRNA, but not *NKX3.1* mRNA, is increased by hypoxia even in the absence of DHT [17]. Thus, the expression of *PSA* mRNA is increased in response to androgen alone, hypoxia alone, and both androgen and hypoxia. In fact, the promoter region of the *PSA* gene has not only multiple AREs [21], but also a functional HRE to which HIF-1 binds in response to hypoxia [17]. On the other hand, hypoxia results in no induction of *NKX3.1* mRNA expression in the presence (10 nM) or absence of DHT [17]. These results, taken together with our present data regarding the effect of hypoxia on AR transactivation (Fig. 1), indicate that hypoxia activates the AR signaling pathway through ARE at low DHT concentrations that mimic the castration-resistant stage, but not at physiological concentrations of DHT.

Hypoxia induces HIF-1α protein expression, and castration-resistant prostate tumors highly express HIF-1α protein [15].

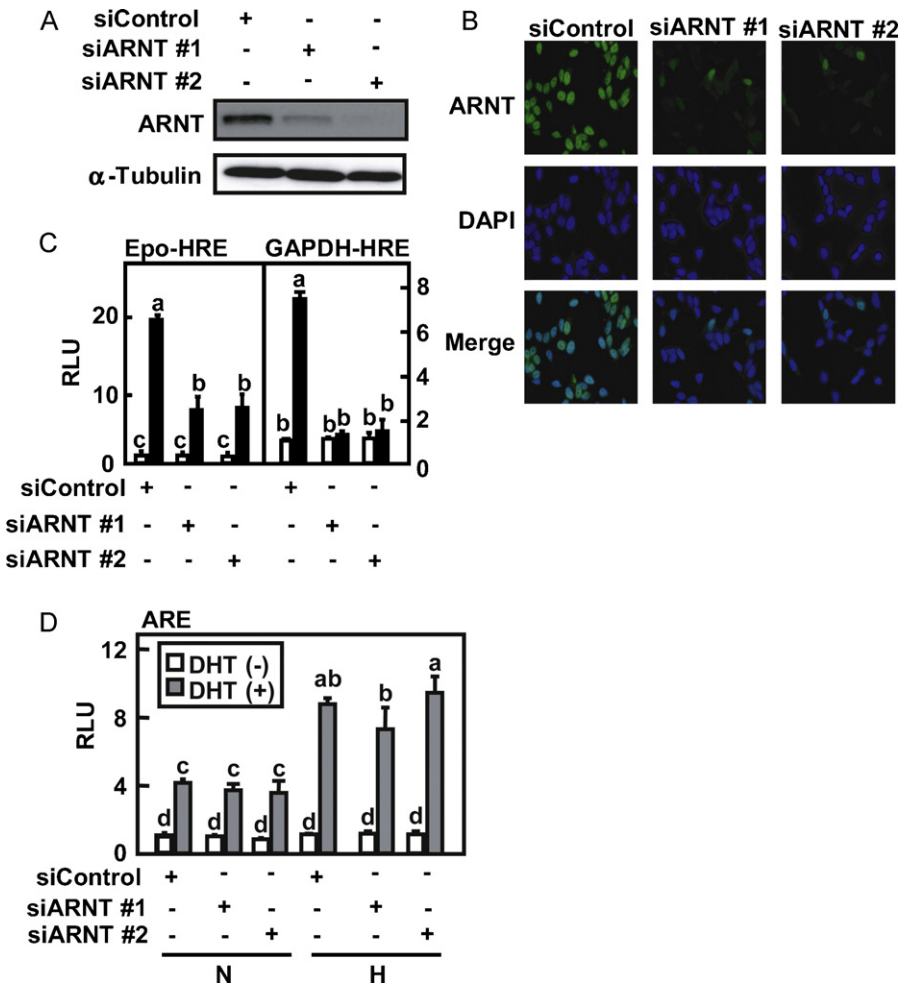

Fig. 6. Effects of HIF-1β/ARNT silencing on hypoxia-enhanced AR transactivation. (A) LNCaP cells were treated with 20 nM HIF-1β/ARNT siRNA (siARNT #1 or siARNT #2) or control siRNA (siControl) for 6 h, followed by incubation in fresh medium for an additional 51 h. Cell lysates were prepared and analyzed by Western blot analyses using anti-ARNT and anti-α-tubulin antibodies. (B) After HIF-1β/ARNT siRNAs treatment, cells were cultured for 51 h. Cells were fixed, permeated, and incubated with anti-ARNT IgG. Immunoreactive HIF-1β/ARNT was visualized using Alexa Fluor 488-conjugated secondary anti-mouse IgG. The nuclear chromatin was stained with DAPI, and random fields were photographed. Quantification of cells stained by DAPI or immunoreacted with anti-ARNT antibodies was performed. Values are calculated from data of random fields, and results are representative of two independent experiments. (C) After HIF-1β/ARNT siRNA treatment, cells were further transfected with pEpo-HRE-Luc (left panel) or pGAPDH-HRE-Luc (right panel) for 24 h, followed by incubation in normoxia (white bars) or hypoxia (black bars) for an additional 9 h. (D) After treatment by siRNA, cells were transfected with pcDNA3.1-AR, pARE-Luc, and pRL-SV40 for 24 h, followed by incubation in the presence and absence of 0.1 nM DHT in normoxia (N) or hypoxia (H) for an additional 9 h. In (C) and (D), luciferase activities were determined. Data are expressed as means ± SD ($n = 3$). The result is representative of three independent experiments. Statistically significant differences ($p < 0.05$) are indicated by different letters.

Knockdown of HIF-1α inhibited hypoxia-enhanced AR transactivation, and exogenous overexpression of a siRNA-resistant form of HIF-1α (HIF-1α(mut)) rescued HIF-1α siRNA-repressed AR transactivation in hypoxia. Furthermore, HIF-1α siRNA inhibited an increase of hypoxia-induced *NKX3.1* mRNA expression in the presence of DHT (0.1 nM). These results indicate that the mechanism by which hypoxia enhances AR transactivation at low DHT levels requires HIF-1α. Furthermore, in two HIF-1α mutants, HIF-1α(A26E) and HIF-1α(R30A), HIF-1α siRNA-suppressed AR transactivation in hypoxia was restored. These mutations result in inactivation of HIF-1 activity due to loss of DNA-binding activity [23]. Thus, these results indicate that HIF-1α enhances AR transactivation at low concentrations of DHT without binding to HREs and that the hypoxic AR transactivation is independent of HIF-1 activity.

HIF-1α forms a heterodimer with HIF-1β/ARNT, and the resultant HIF-1 exerts its activity through binding to HRE in hypoxia-inducible genes [14]. HIF-1α^{516} forms a complex with HIF-1β/ARNT in the cytosol and appears to inhibit the nuclear translocation of HIF-1β/ARNT [20]. Because HIF-1β/ARNT is an essential factor for HIF-1α-dependent HIF-1 activity, HIF-1α^{516} functions as a dominant negative form for HIF-1 activity. Exogenous expression of HIF-1α^{516} inhibited hypoxia-activated AR transactivation, suggesting that HIF-1α^{516} inhibits the activating effect of HIF-1α on AR transactivation by interrupting interaction between HIF-1α and HIF-1β/ARNT. However, two types of HIF-1β/ARNT siRNA, which are effective at inhibiting HIF-1 activity on HRE reporters, had no influence on hypoxia-activated AR transactivation, indicating that HIF-1α^{516} inhibits hypoxia-enhanced AR transactivation through a mechanism that does not require HIF-1β/ARNT. Furthermore, HIF-1α(mut) had no influence on hypoxia-activated AR transactivation (Fig. 2E), suggesting that HIF-1α is necessary but not sufficient for AR transactivation in hypoxia. Thus, HIF-1α promotes AR function through a novel mechanism different from the HIF-1 signaling pathway in hypoxia at low levels of DHT. HIF-1α may need to interact with certain proteins other than HIF-1β/ARNT to activate AR function.

The present data provide direct evidence that hypoxia induces HIF-1α-mediated AR transactivation independent of HIF-1 activity. On the other hand, Park et al. [25] reported that hypoxia for

4 h enhances AR transactivation and induces binding of AR to the ARE in LNCaP cells and that reoxygenation for 2 h after hypoxic treatment (hypoxia-reoxygenation) further enhances AR transactivation and results in higher ARE-binding activity. However, hypoxia increases HIF-1α protein expression, and hypoxia-reoxygenation decreases HIF-1α protein expression. Therefore, they suggest that HIF-1 signaling is unlikely involved in the mechanisms of AR stimulation by hypoxia-reoxygenation. Taken together, although HIF-1 activity is not required for AR transactivation during hypoxia and hypoxia-reoxygenation, HIF-1α is involved in AR function during hypoxia, but not during reoxygenation. Thus, the mechanisms by which hypoxia alone and reoxygenation after hypoxia enhance AR transactivation are different. Furthermore, hypoxia-reoxygenation enhances AR transactivation at low concentrations of androgens [25]. Here we demonstrated that hypoxia alone increased AR transactivation at low DHT levels, indicating that hypoxia itself has an effect on sensitization of AR to androgens although it remains unclear whether reoxygenation affects the sensitization.

Hypoxia caused increased AR transactivation in the presence of DHT (0.1 nM), but not in the absence of DHT. Likewise, hypoxia-reoxygenation has no effect on AR transactivation in the absence of androgen [25]. In contrast, Khandrika et al. [26] analyzed the transcriptional activity of AR using a reporter vector containing PSA enhancer (−4758 to −3884) and reported that hypoxia activates AR transactivation in the absence of DHT. PSA gene has a HRE between −3951 and −3947, and hypoxia increases the promoter activity of PSA gene in androgen-independent prostate cancer cells [17], consistent with the present results that hypoxia increased PSA mRNA in the absence of DHT. These results indicate that neither hypoxia nor reoxygenation activates AR transactivation in the absence of androgen.

Hypoxia induces the phosphorylation levels of p38 mitogen-activated protein kinase (MAPK) and HSP27 and increases the expression levels of AR and HIF-1α [26]. Furthermore, SB203580, which is an inhibitor for p38 MAPK, suppresses the expression levels of hypoxia-increased AR, HIF-1α, and phospho-HSP27 and results in decreased transcriptional activities of AR and HIF-1α. Because cooperative interaction between ligand-activated AR and phospho-HSP27 increases AR stability, shuttling, and transcriptional activity [27], Khandrika et al. [26] suggest that AR is activated by p38 MAPK and HSP27 in hypoxia and that activated P38 MAPK stabilizes HIF-1α and promotes HIF-1-mediated gene transcription. Taken together with the fact that HIF-1α acted on AR function independent of HIF-1 activity at low DHT levels mimicking castration-resistant stage in hypoxia, SB203580 may suppress hypoxia-enhanced AR function, in addition to HIF-1 activity, by inhibiting HIF-1α expression.

The AR signaling pathway plays a dominant role in the progression of CRPC. The present study reveals that the HIF-1α-mediated crosstalk between the AR signaling and hypoxic signaling pathways contributes to AR transactivation in CRPC. HIF-1α may characterize the phenotype of aggressive CRPC and therefore it might be a molecular target for a chemopreventive and/or chemotherapeutic agent to repress the AR signaling in CRPC.

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