Androgen-Dependent Gene Expression of Prostate-Specific Antigen Is Enhanced Synergistically by Hypoxia in Human Prostate Cancer Cells

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Abstract
The androgen receptor (AR) is implicated in prostate cancer growth, progression, and angiogenesis. Hypoxia-inducible factor-1 (HIF-1), which transcriptionally regulates hypoxia-inducible angiogenic factors, is up-regulated in prostate cancers compared with adjacent normal tissues. HIF-1 may be involved in prostate cancer as well as the AR, but the involvement of HIF-1 in prostate cancer angiogenesis and progression has not been fully elucidated. In the present study, we found that in prostate cancer LNCaP cells, dihydrotestosterone enhanced the expression of GLUT-1, one of the HIF-1 target genes, and also that hypoxia enhanced the expression of prostate-specific antigen (PSA) that is one of the AR target genes and is involved in tumor invasion. Small interfering RNA that specifically inhibits HIF-1 reduced the expression levels of PSA as well as GLUT-1. Reporter gene analysis showed that dihydrotestosterone activated the HIF-1–mediated gene expression and hypoxia enhanced the AR-induced promoter activity of human PSA gene. Deletion and site-directed mutation of the 5′-flanking region of human PSA gene revealed that the sequence ACGTG between −3951 and −3947 was essential in the response to hypoxia. Furthermore, chromatin immunoprecipitation assay indicated that HIF-1 interacts with the AR on the human PSA gene promoter. These results indicate that in prostate cancers, HIF-1 might cooperate with the AR to activate the expression of several genes related to tumor angiogenesis, invasion, and progression. (Mol Cancer Res 2007;5(4):383–91)

Introduction
Prostate cancer is the most common malignancy and the second most frequent cause of cancer-related death of men in the United States (1). Several advances have been made recently to elucidate the mechanism by which androgen regulates prostate cancer growth through the androgen receptor (AR; refs. 2, 3). The AR is found to be expressed in the majority of prostate cancer samples, both the primary and metastatic sites, and also including those with androgen-refractory status. The AR activates gene expression that is involved in the growth, differentiation, and maintenance of prostate epithelium. Prostate-specific antigen (PSA), a chymotrypsin-like serine protease, is a well-known AR-regulated gene in the human prostate gland and is often increased in hyperplastic and inflammatory lesions (4, 5). PSA has the ability to cleave proteins that affect cell migration and metastasis. Currently, serum PSA is widely used as a marker for the diagnosis of prostate cancer and disease progression. Upon binding to androgen, the AR translocates into the nucleus and binds to the androgen responsive elements (ARE) on the PSA promoter. In addition to the PSA gene, the expression of several other genes has been found to be up-regulated via AR-mediated signaling in prostate cancer (3). Prostate cancer therapy is dependent on the stages of the tumor and AR expression. Early stage androgen-responsive prostate cancer can be treated by castration or with antiandrogens or drugs that block androgen-induced responses (6).

The growth of prostate cancer, as well as the other cancers, is dependent on blood supply and the induction of new blood vessels from preexisting ones through angiogenesis (7). Tumor-induced angiogenesis is regulated by cell-produced factors that have mitogenic and chemotactic effects on vascular endothelial cells. Vascular endothelial growth factor (VEGF) plays a central role in tumor-induced angiogenesis (8, 9). The elevated expression of VEGF has been found in human and animal models of prostate cancer (10, 11). Tumor hypoxia is thought to be the most potent stimulus for VEGF (12-14), and its expression is transcriptionally regulated by hypoxia-inducible factor-1 (HIF-1; refs. 15, 16).

HIF-1 is a heterodimer composed of HIF-1α and HIF-1β, both of which belong to bHLH/PAS domain transcriptional factors (17). The HIF-1β subunit is identical to the
arylhydrocarbon receptor nuclear translocator, which also serves as a heterodimeric partner with the arylhydrocarbon receptor. In contrast, it seems that the sole but critical function of the HIF-1α subunit is to mediate the response to hypoxia. Under normoxic conditions, the HIF-1α subunit is rapidly degraded via the ubiquitin-proteasome pathway triggered by oxygen-dependent hydroxylation of proline residues that occurs in oxygen-dependent degradation (18, 19). It has been reported that the von Hippel-Lindau tumor-suppressor protein recognizes these hydroxylated proline residues and then binds to the HIF-1α subunit, resulting in its proteasome degradation (20). Under hypoxic conditions, this oxygen-dependent degradation system is repressed, and then stabilization of the HIF-1α subunit initiates a multistep pathway of activation of HIF-1α. This pathway includes hypoxia-dependent nuclear translocation and dimerization with arylhydrocarbon receptor nuclear translocator, bringing about an interaction with the hypoxia responsive element (HRE) of target genes, including VEGF, followed by recruitment of transcriptional activators.

Although it was shown that androgens regulate VEGF content in normal and malignant prostate cells (21-24), the mechanisms of this process have not been clearly defined. Mabjeesh et al. (25) recently showed that androgens activate HIF-1, driving VEGF expression in androgen-sensitive LnCaP cells. It was also shown that HIF-1 is up-regulated in prostate cancer tissues compared with adjacent normal tissues (26, 27). However, the involvement of HIF-1 in prostate cancer angiogenesis and the effect of HIF-1 on AR-mediated gene expression have not been fully elucidated. In the present study, we investigated the cross talk between the AR and HIF-1 in prostate cancer. We found that the AR enhances HIF-1-mediated gene expression and that HIF-1 interacts with the AR on the PSA gene promoter and activates its expression.

Results

Effects of Androgen and Hypoxia on HIF-1- and AR-Mediated Gene Expression in LnCaP Cells

We first examined the effect of dihydrotestosterone on GLUT-1 gene expression and the effect of hypoxia on PSA gene expression in human prostate cancer LnCaP cells. It has previously been shown that the expressions of GLUT-1 and PSA are induced by the activation of HIF-1 and the AR, respectively (3, 4, 18, 19). As indicated in Fig. 1A, the effects of dihydrotestosterone and hypoxia on gene expression were synergistic. Dihydrotestosterone enhanced hypoxia-induced GLUT-1 gene expression. Hypoxia stimulated dihydrotestosterone-induced PSA gene expression. Furthermore, small interfering RNA (siRNA) to specifically inhibit HIF-1 reduced the expression levels of PSA as well as those of GLUT-1 (Fig. 1B).

To investigate the effect of hypoxia on the promoter activity of the PSA gene, we cloned 4.5 kb of the 5′-flanking region of the human PSA gene (−4457 to +12), constructed the pPSA4.5-Luc reporter plasmid, and then did transient transfection analysis using pPSA4.5-Luc plasmid in LnCaP cells. The 5′-flanking region of human PSA gene (−4457 to +12) has multiple AREs (28). As shown in Fig. 2A, the activity of pPSA4.5-Luc was enhanced by dihydrotestosterone, and hypoxia synergistically enhanced the dihydrotestosterone-induced promoter activity. On the other hand, hypoxia did not enhance the transcriptional activity of the androgen-responsive reporter plasmid pARE-Luc containing two copies of ARE (ref. 29; Fig. 2B). As shown in Fig. 1C, except PSA, hypoxia did not affect the expression of AR-responsive genes such as NKX3.1 (30) and PMEP1A (31). These results indicated that the enhancement of PSA expression by hypoxia might be PSA gene specific. HIF-1 induced by hypoxia might act on a region of the PSA gene promoter other than ARE to enhance the expression of PSA in LnCaP cells.

**FIGURE 1.** Synergistic effects of androgen and hypoxia on the expressions of PSA and GLUT-1 in LnCaP cells. A and C, LnCaP cells were precultured in DMEM containing 10% charcoal-stripped serum for 2 d and then cultured under hypoxia (H, 1% O2) or normoxia (N, 21% O2) with (+) or without (−) 10 nmol/L dihydrotestosterone (DHT) for 20 h. Total RNAs were extracted from these cells, and the extracted RNAs (30 μg) were analyzed by Northern blot analysis using 32P-labeled DNA probe for PSA, GLUT-1 (A), NKX3.1, and PMEP1A (C). The abundance of β-actin mRNA was also analyzed as an internal standard. Relative mRNA levels were determined by dividing the levels of nontreated cells. B, LnCaP cells were transfected with 100 nmol/L of HIF-1α (+) or control siRNA (−). After 24 h, cells were cultured under hypoxia or normoxia with (+) or without (−) 10 nmol/L dihydrotestosterone for 20 h. HIF-1α protein levels were measured by Western blot analysis using anti-HIF-1α antibody. PSA, GLUT-1, and β-actin mRNA levels were measured by Northern blot analysis. Relative mRNA levels were determined by dividing the levels of control siRNA-transfected nontreated cells.

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Furthermore, we examined the effect of dihydrotestosterone on the activity of HIF-1 by reporter gene analysis using the hypoxia-responsive reporter plasmid pHRE-Luc containing six copies of HRE (32). Our results showed that dihydrotestosterone enhanced hypoxia-inducible HRE-mediated transcription in both LNCaP cells (Fig. 3A) and AR-transfected COS-7 cells (Fig. 3B). The protein level of α-subunit of HIF-1 was also up-regulated by dihydrotestosterone (Fig. 1B). Mabjeesh et al. (25) recently showed that dihydrotestosterone activated VEGF expression in LNCaP cells. We confirmed that dihydrotestosterone enhanced hypoxia-induced VEGF expression (data not shown). The HIF-1/HRE-mediated expression of genes other than GLUT-1 might be also be enhanced by treatment with dihydrotestosterone.

**Effect of Hypoxia on the Promoter Activity of the PSA Gene in AR-Transfected PC-3 Cells**

We next investigated the effect of hypoxia on the promoter activity using AR-transfected PC-3 cells. PC-3 cells are derived from human prostate cancer as well as LNCaP cells but do not express the AR at the mRNA level (33). In mock- or HIF-1α-transfected cells, the promoter activity of the pPSA4.5-Luc plasmid was slightly increased by hypoxia but not affected by dihydrotestosterone (Fig. 4). On the other hand, AR-transfected PC-3 cells could respond to dihydrotestosterone, and in the AR-transfected cells hypoxia enhanced the dihydrotestosterone-responsive promoter activity of the pPSA4.5-Luc plasmid. Cotransfection of the AR with HIF-1α markedly enhanced the additive effect of hypoxia and dihydrotestosterone. These results indicated that the AR and HIF-1 interact with each other and then cooperatively increase the promoter activity of PSA gene in prostate cancer.

**Identification of the Hypoxia-Responsive Region in the Human PSA Promoter**

To examine which region is functional for the hypoxia-inducible promoter activity of the human PSA gene, we next constructed the deletion mutants of pPSA4.5-Luc reporter plasmids (Fig. 5A) and then did transient transfection analysis.
using these plasmids. As shown in Fig. 5B, the dihydrotestosterone and hypoxia-inducible promoter activities of the deletion mutants, pPSA/D1-Luc and pPSA/D3-Luc plasmids, were roughly similar to the activities of the pPSA4.5-Luc plasmid. On the other hand, the promoter activity of the pPSA/D2-Luc plasmid was not enhanced by hypoxia, although its activity was increased by dihydrotestosterone. Furthermore, we constructed the deletion mutants of pPSA/D3-Luc reporter plasmids and found that the promoter activity of the pPSA/D4-Luc plasmid was enhanced by hypoxia but that the promoter activity of the pPSA/D5-Luc plasmid was not (Fig. 5C). These results indicated that the region −3963 to −3932 has hypoxia-inducible promoter activity.

The region of human PSA gene −3963 to −3932 contains the core recognition sequence (5′-RCGTG-3′) necessary for the binding of HIF-1 (34) between −3951 and −3947 (Fig. 5A). We therefore tested the involvement of this sequence in the PSA gene expression using the site-specific mutant pPSA/M6-Luc reporter plasmid. As shown in Fig. 5C, no significant enhancement of the promoter activity by hypoxia was observed. We further examined the effect on PSA promoter activity of a constitutively active mutant of HIF-1α (CA HIF-1α) that had been generated by the substitution of proline 564 and asparagine 803 for alanine residues (35). The overexpression of CA HIF-1α, as well as hypoxic conditions, enhanced the promoter activity of the pPSA/D4-Luc plasmid but did not affect the promoter activity of the pPSA/M6-Luc plasmid (Fig. 5D). These results indicated that the sequence ACGTG between −3951 and −3947 in the human PSA gene promoter functions as HRE and is essential for HIF-1 binding in response to hypoxia.

Cross Talk between the AR and HIF-1 in Prostate Cancer Cells

The observed synergistic activation of the PSA promoter by the AR and HIF-1 implies a physical interaction between these proteins. To verify this interaction, we attempted to endogenously coimmunoprecipitate the AR and HIF-1α. As shown in Fig. 6A, when LNCaP cells were cultured under hypoxia in the presence of 10 nmol/L dihydrotestosterone, HIF-1α and the AR were communoprecipitated with each other, indicating a potential physical interaction between HIF-1 (HIF-1α/arylhydrocarbon receptor nuclear translocator complex) and the AR.

Human PSA gene promoter has multiple AREs, and one of these AREs (designated as ARE III; ref. 28) locates near the HRE (−3951 to −3947) on the promoter (Fig. 6B). To investigate if HIF-1 and the AR physically interact on the PSA promoter, we next did a chromatin immunoprecipitation (ChIP) assay using chromatin isolated from LNCaP cells. As shown in Fig. 6C, PCR analysis of the immunoprecipitates in samples derived from dihydrotestosterone-treated cells that had been isolated using an anti-AR antibody revealed that the ARE III of the PSA gene was associated with the AR. Furthermore, we found that both the ARE III− and HRE-containing sequences of the PSA promoter were recovered in anti-AR immunoprecipitates from dihydrotestosterone and hypoxia-treated cells. It was noteworthy that the ARE III was recovered higher in the precipitates from dihydrotestosterone and hypoxia-treated cells than those from the cells treated only with dihydrotestosterone. These results indicated that HIF-1 interacts with the AR on this region in the human PSA gene promoter to activate its expression in LNCaP cells cultured with dihydrotestosterone under hypoxia.

Discussion

In the present study, we investigated the cross talk between the AR and HIF-1 in prostate cancer cells and found that hypoxia and androgen synergistically activated AR- and HIF-1−dependent gene expression (Figs. 1-4).

In LNCaP cells, hypoxia stabilizes HIF-1α protein and then forms the complex of HIF-1 and the AR on the human PSA gene promoter to activate its expression (Fig. 6). Because hypoxia did not affect the AR-dependent reporter gene expression (Fig. 2), HIF-1 might not affect the transcriptional activity of the AR on the different gene promoter, but it might increase the activity of the AR that is bound to the site close to the HIF-1 binding site. Fei et al. (36) reported that HIF-1 interacts with p53 on the Bnip3L gene promoter to activate its p53-dependent gene expression. They indicated that the
transcriptional coactivator CBP is recruited to the region near the p53-binding site through the action of HIF-1, which is activated under hypoxia and bound to the HIF-1 binding site on the Bnip3L gene promoter and that then p53 can be more efficiently recruited to the region.

Furthermore, Sánchez-Elsner et al. (37, 38), who reported that hypoxia and transforming growth factor-β synergistically activate the expressions of VEGF and erythropoietin through the formation of the complex of HIF-1 with Smads on their genes, suggested that transforming growth factor-β–activated Smad might stabilize the multifactorial complex composed of HIF-1 and the coactivator to activate their gene expression. Our ChIP analysis showed that the ARE III–containing sequences of the PSA promoter were recovered higher in anti-AR immunoprecipitates from dihydrotestosterone and hypoxia-treated cells than from the cells treated only with dihydrotestosterone. On the PSA gene promoter, HIF-1 might stabilize the transcriptional complex containing the AR and increase the recruitment of the coactivator to the AR. As shown in Fig. 1C, hypoxia did not affect the expression of two of the other AR-responsive genes, NKX3.1 (30) and PMEP1 (31), and we had not found other AR-regulated genes containing putative HREs. However, HIF-1 may be involved in AR-mediated gene expression other than PSA in the same machinery.

PSA can cleave proteins that affect cell migration and metastasis (3). For instance, PSA has the capacity to cleave extracellular matrix glycoproteins such as fibronectin and laminin and facilitate prostate cancer cell invasion (39). PSA can cleave and activate the urokinase-type plasminogen activator, which enhances tumor cell invasion (40). In prostate cancers, HIF-1 might cooperate with the AR to activate the expression of PSA and then facilitate prostate cancer invasion and progression.

Furthermore, we here found that androgen increases the protein level of HIF-1α and then enhances HIF-1–dependent reporter gene expression (Figs. 1 and 3). The effect of androgen on HIF-1–dependent gene expression was different from the effect of hypoxia on AR-dependent gene expression, which is PSA gene specific. Androgen might increase the expression of many hypoxia-inducible genes involved in cancer growth, progression, and angiogenesis such as VEGF (18). The clinical

**FIGURE 5.** Identification of the hypoxia-responsive region on the human PSA gene promoter. A. Reporter plasmids containing the 5’-flanking region of the human PSA gene with deletion or site-specific mutation are represented schematically. Closed boxes, ARE I, II, and III identified by Cleutjens et al. (3). Shaded box, putative HRE. B and C. LNCaP cells were transiently transfected with pRL-SV40 and deleted or site-specific mutated reporter plasmids. The transfected cells were cultured under hypoxia (1% O2; closed columns) or normoxia (21% O2; open columns) with (+) or without (−) 10 nmol/L dihydrotestosterone for 20 h. Cell lysates were subjected to dual luciferase assays and determined by dividing the normalized luciferase activity from nontreated cells. Results are the average of three independent experiments. Columns, mean; bars, SD. *, P < 0.01 versus cells cultured under normoxia. D. LNCaP cells were transiently transfected with pRL-SV40, reporter plasmids (pPSA/D4-Luc or pPSA/M6-Luc), and empty vector (open columns) or CA HIF-1α expression vector (shaded columns). The transfected cells were cultured in the presence (+) or absence (−) of 10 nmol/L dihydrotestosterone for 20 h. Cell lysates were subjected to dual luciferase assays and determined by dividing the normalized luciferase activity from mock-transfected nontreated cells. Results are the average of three independent experiments. Columns, mean; bars, SD. *, P < 0.01 versus mock-transfected cells.
antineoplastic activity of antiandrogen on prostate cancers might in part involve reduction of the HIF-1α-dependent gene expression. The activation of HIF-1α–dependent gene expression by androgen was recently reported by Mabjeesh et al. (25). They reported that in LNCaP cells cultured under normoxia, androgen increases the secretion of epidermal growth factor and that the epidermal growth factor activates HIF-1α–dependent gene expression by enhancement of the synthesis of the HIF-1α protein through an autocrine loop mechanism involving the phosphatidylinositol 3-kinase/AKT/FRAP pathway in LNCaP cells.

Here, we showed that the androgen-activated AR not only activates HIF-1α–dependent gene expression under normoxic conditions in the autocrine loop mechanism as described by Mabjeesh et al. (25) but also physically interacts with HIF-1α and then activates HIF-1α–dependent gene expression under hypoxic conditions (Figs. 3 and 6). The effect of dihydrotestosterone on hypoxia-inducible HRE-mediated transcription was also observed in AR-transfected COS-7 cells, which does not secrete epidermal growth factor in response to dihydrotestosterone (Fig. 3B). Because the protein level of HIF-1α is up-regulated...
by dihydrotestosterone (Fig. 1B), the AR might stabilize HIF-1α protein through their physical interaction. Kodama et al. (41) also reported that under hypoxic conditions, HIF-1–dependent gene expression is further up-regulated by glucocorticoid by the glucocorticoid receptor. Stabilized HIF-1α protein might interact with several activated nuclear receptors and then be activated by their interactions. The commercial anti–HIF-1α antibody, which we used for immunoprecipitation analysis shown in Fig. 6A, had been raised against residues 329 to 530 of human HIF-1α. It was noteworthy that the other anti–HIF-1α antibody, raised against residues 618 to 637 of human HIF-1α that locates near the NH2-terminal transactivation domain of HIF-1α (32), failed to coimmunoprecipitate the AR even when LNCaP cells were cultured under hypoxia in the presence of 10 nmol/L dihydrotestosterone (data not shown).

Kodama et al. (41) showed that transactivation domain of HIF-1α was necessary for enhancement of HIF-1–dependent gene expression by glucocorticoid using deletion mutants of HIF-1α. Activated nuclear receptors might interact with the transactivation domain of HIF-1α mediated by transcriptional coactivators and enhance HIF-1–dependent gene expression.

In summary, we found that in prostate cancers, the AR enhances HIF-1–mediated gene expression and that HIF-1 interacts with the AR on the PSa gene promoter and activates its expression. HIF-1 might be involved in AR-mediated gene expression other than PSa in prostate cancer and implicated in prostate cancer growth and progression. HIF-1 may be a prime target for prostate cancer therapy.

Materials and Methods

Cell Culture

Human prostate cancer LNCaP and PC-3 cells and COS-7 cells were cultured and maintained in DMEM containing 10% FCS. Our examination of the effects of dihydrotestosterone on these cells used cells cultured in DMEM supplemented with 10% FCS that had been treated with charcoal (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) before use.

Plasmids

The 5′-flanking region of the human PSa gene, which covered the nucleotide positions −4457 to +12 from transcription start site (42) as +1, was amplified by PCR from human genomic DNA using two primers: PSA-Fw (5′-TTCGAAGATCTCTCATGCTGTGCA-3′; introduced XhoI sites being shown in small letters) and PSA-Rv (5′-TGTTAAGCTTGGGCTGGAGGCCTC-3′). The amplified fragment was then ligated into the XhoI/HindIII site of the pGL3-basic vector (Promega, Madison, WI) to produce the reporter plasmid pPSA4.5-Luc. The pPSA/D1-Luc vector was constructed by ligating the PCR products using two primers, PSA/D1-Fw (5′-ATGCAAGATCTATGCTGTGCA-3′) and PSA/D1-Rv (5′-TGTTAAGCTTGGGCTGGAGGCCTC-3′), into the XhoI/EcoRV digested pPSA4.5-Luc. The pPSA/D2-Luc vector was constructed by digesting the pPSA4.5-Luc with EcoRV and AarII. The pPSA/D3-Luc vector was constructed by digesting the pPSA4.5-Luc with BamHI and BgII. The pPSA/D4-Luc, the pPSA/D5-Luc, and the pPSA/M6-Luc vectors were constructed by ligating the PCR products using two primers, PSA-Fw and PSA/D4-Rv (5′-AAAGATCTCTCATGCTGTGCA-3′); PSA/D5-Rv (5′-AAAGATCTCTCATGCTGTGCA-3′); and PSA/M6-Rv (5′-AAAGATCTCTCATGCTGTGCA-3′); mutated nucleotides being shown in small letters) into the XhoI/BglII–digested pPSA4.5-Luc. All plasmids constructed as described above were verified by sequencing. The expression plasmids for human HIF-1α and the AR, the hypoxia-responsive reporter plasmid pHRE-Luc, and the androgen-responsive reporter plasmid pARE-Luc have been previously described (29, 32).

RNA Isolation and Northern Blot Analysis

LNCaP cells were cultured in DMEM containing 10% charcoal-stripped FCS for 2 days and treated with or without 10 nmol/L dihydrotestosterone under hypoxia (1% O2) or normoxia (21% O2) for 20 h. Total RNA from the harvested cells was isolated by RNA-Bee (TEL-TEST, Inc., Friendswood, TX). Twenty micrograms of total RNA were denatured with formamide, electrophoresed on a 1% agarose gel, and transferred to a PhotoGene nylon membrane (Life Technologies, Inc., Rockville, MD). The blots were hybridized with cDNAs for GLUT-1 (43), PSA (29), NXX3.1, PMEP1, or β-actin. The mRNA levels were calculated on the basis of hybridization signals measured by a Fujix Bio-imaging analyzer BAS2000 (Fuji Photo Film Co., Tokyo, Japan).

siRNA Duplexes and Treatment

The HIF-1α siRNA duplexes targeted nucleotides 1521 to 1541 of the HIF-1α mRNA sequence and composed of sense 5′-CUGAGUAGACCGAUCUGAdTdT-3′ and antisense 5′-UCAAAGUUUCGCUGGCAUCAdTdT-3′. The control siRNA duplex did not target any gene and is composed of sense 5′-AGUUACAGCCAGGUAUAGdTdT-3′ and antisense 5′-GACUACUGGUGUAGUACAdTdT-3′. LNCaP cells were transfected with 100 nmol/L HIF-1α or control siRNA by the Oligofectamine method according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA).

Western Blot Analysis

LNCaP cells were seeded and cultured for 2 days and then treated with or without 10 nmol/L dihydrotestosterone under hypoxia or normoxia for 20 h. Total cell extracts were prepared and resolved on a 7% SDS-polyacrylamide gel. After electrophoresis, the proteins were electrotransferred to a polyvinylidene difluoride membrane, probed with rabbit polyclonal anti–HIF-1α antibody (32), and detected by chemiluminescence.

Reporter Gene Assay

For transfection into LNCaP cells, the cells were transiently transfected with 2 μg of the luciferase reporter plasmid [pHRE-Luc (32), pARE-Luc (29), or PSA promoter-Luc] and 0.4 μg of the Renilla luciferase-expressing plasmid pRL-SV40 (as an internal transfection efficiency control) by the LipofectAMINE method according to the manufacturer’s instructions (Invitrogen). After 6 h of incubation with the LipofectAMINE-DNA complex, the cells were washed and
cultured for 18 h and then cultured under normoxia (21% O₂) or hypoxia (1% O₂) with or without dihydrotestosterone for an additional 20 h. For transfection into PC-3 and COS-7 cells, the cells were transiently transfected by the Lipofectamine 1 plasmid, and 0.04 AMINE with 1 g of the pRL-SV40 plasmid. After 16 h of incubation with the LipofectAMINE-DNA complex, the cells were washed and cultured for 8 h and then cultured under normoxia (21% O₂) or hypoxia (1% O₂) with or without dihydrotestosterone for an additional 20 h. The cells were then lysed and assayed for firefly and Renilla luciferase activities using the Dual-Luciferase Reporter Assay system (Promega).

Immunoprecipitation

LNCaP cells were seeded in 10-cm-diameter culture plates and grown until they were 50% confluent, cultured with a medium containing 10% charcoal-stripped FCS for 2 days, and then treated with or without 10 nmol/L dihydrotestosterone under hypoxia or normoxia for 20 h. Following these treatments, the cells were harvested and lysed in immunoprecipitation buffer (20 mmol/L sodium phosphate, 500 mmol/L NaCl, 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate, and 0.02% sodium azide). Samples were subjected to immunoprecipitation with anti-AR (Affinity BioReagents, Inc., Golden, CO) or anti–HIF-1α (Stressgen Bioreagents, Ann Arbor, MI) antibodies overnight at 4°C with gentle rotation. Then, the immunocomplexes were incubated with protein A or G agarose, washed with immunoprecipitation buffer, and then eluted with SDS sample buffer (2% SDS, 62.5 mmol/L Tris-HCl, 10% glycerol, 2% mercaptoethanol). Immunoprecipitates were resolved on a 7% SDS-PAGE and then visualized using anti-AR or anti–HIF-1α antibody, as described in the Western Blot Analysis section.

ChIP Assay

The ChIP assay was done according to the Upstate, Inc. (Charlottesville, VA), protocol. LNCaP cells were seeded in 15-cm-diameter culture plates and grown until they were 50% confluent, cultured in DMEM containing 10% charcoal-stripped FCS for 2 days, and then treated with or without 10 nmol/L dihydrotestosterone under hypoxia or normoxia for 20 h. Following these treatments, the cells were fixed by 1% formaldehyde. After cross-linking, the cells were rinsed with ice-cold PBS and harvested with SDS lysis buffer. This was followed by sonication. Samples were diluted with ChIP dilution buffer and precleared with protein A agarose, followed by incubation with anti-AR antibody overnight at 4°C with gentle rotation. Immunocomplexes were recovered by protein A agarose for 2 h at 4°C and then were washed five times. Immunocomplexes were eluted with 1% SDS in 0.1 mol/L NaHCO₃, and cross-linking was reversed by heating to 65°C. DNAs were purified by proteinase K digestion, phenol-chloroform extraction, and ethanol precipitation. The specific promoter region was amplified by PCR for PSA promoter region –3986 to –3812 as follows: HRE, sense 5'-AGGTGTGCTGTTTGCCTAC-3' and antisense 5'-TTATGCTAAAACGAGTTTCT-3'.

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References

Molecular Cancer Research

Androgen-Dependent Gene Expression of Prostate-Specific Antigen Is Enhanced Synergistically by Hypoxia in Human Prostate Cancer Cells

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