Androgens Stimulate Hypoxia-inducible Factor 1 Activation via Autocrine Loop of Tyrosine Kinase Receptor/Phosphatidylinositol 3’-Kinase/Protein Kinase B in Prostate Cancer Cells

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Abstract

Purpose: Androgen deprivation is implicated in reducing neoangiogenesis in prostate cancer (PCA). Androgens regulate the expression of the vascular endothelial growth factor (VEGF); hypoxia stimulates VEGF expression through the activation of the transcriptional factor, hypoxia-inducible factor 1 (HIF-1). We tested the hypothesis that an effect of androgens on VEGF expression is regulated directly by HIF-1 and HIF-2, and antiandrogens block HIF function.

Experimental Design: Androgen and antiandrogen effects were evaluated on HIF-1α protein and HIF-1 transcriptional activation in human PCA cells.

Results: Dihydrotestosterone (DHT) activates HIF-1α nuclear protein expression in LNCaP cells but not in androgen-receptor-negative PC-3 cells. HIF-1α expression is correlated with the transactivation of a hypoxia-responsive element-driven reporter gene and with the production of VEGF protein. The effect of DHT on HIF-1 was blocked by nonsteroidal antiandrogens, flutamide and bicalutamide. DHT does not affect HIF-1α mRNA levels but regulates HIF-1α protein expression through a translation-dependent pathway. PC-3 cells when incubated with increasing amounts of conditioned medium from LNCaP cells treated with DHT experienced a dose-dependent increase in HIF-1α. This induction was not seen either when LNCaP cells were treated with flutamide or conditioned medium were pretreated with antibody to the epidermal growth factor (EGF). HIF-1 activation by DHT was blocked by LY294002, a potent inhibitor of the phosphatidylinositol 3’-kinase signaling pathway, whereas HIF-1 activation by EGF, as ligand, was not inhibited by flutamide. In contrast, HIF-2α protein was not affected by androgens or antiandrogens.

Conclusion: Androgens activate HIF-1, driving VEGF expression in androgen-sensitive LNCaP cells. This regulation is mediated through an autocrine loop involving EGF/ phosphatidylinositol 3’-kinase/protein kinase B, which in turn activate HIF-1α and HIF-1-regulated gene expression. Therapeutic actions of antiandrogens in PCA include inhibition of HIF-1 function.

Introduction

For >60 years androgen deprivation therapies have remained the mainstay of therapy for metastatic PCA.3 Antandrogens have postulated recently to reduce angiogenesis as well as induce apoptosis in PCA (1). More recently, p.o. available nonsteroidal antiandrogens like flutamide and bicalutamide block this axis clinically. The growth of PCA and other tumors is dependent on its blood supply and induction of new blood vessels from pre-existing ones through angiogenesis (2, 3). The discovery of angiogenesis-dependent tumor growth involves the release of soluble factors, including VEGF, transforming growth factor α, platelet-derived growth factor, acidic and basic fibroblast growth factors, insulin-like growth factor I, and matrix metalloproteinases (2, 3). VEGF is a critical one, which can be produced by many tumor cell types (4). Elevated expression of VEGF has been shown in human and animal models of PCA (5, 6). VEGF expression is regulated by various growth factors, cytokines, estrogen, progesterone, and glucocorticoids (7–14). Hypoxia is thought to be the most potent stimulus for VEGF (15–18), and its expression is transcriptionally regulated by HIF-1 (19–22). Androgens regulate VEGF content in normal and malignant prostate cells (23–26). However, the mechanisms underlining the above observations are not clearly defined.

HIF-1 is a critical, genome-wide transcription regulator identified for O₂ homeostasis responsive to hypoxic stress. HIF-1 controls the expression of >40 genes including VEGF, of which the protein products are involved in angiogenesis, erythropoiesis, glycosylation, and invasion (27). HIF-1 is a heterodimer composed of HIF-1α and HIF-1β subunits, which are basic
helix-loop-helix-Per/Amt/Sim domain proteins. HIF-1β is con-
stitutively expressed, whereas the expression of HIF-1α is main-
tained at low levels in most cells under normoxic conditions.
Under hypoxic conditions, HIF-1α escapes proteasomal degra-
dation and then translocates to the nucleus. The former process
results from inhibiting the activity of oxygen-dependent prolyl
hydroxylases that modify residues 564 and 402 (28), and the
latter process is mediated by nuclear localization signals.
This enzymatic modification of HIF-1α is required for the binding of
von Hippel-Lindau protein, which is the recognition component
of an E3 ubiquitin-protein ligase that targets HIF-1α for protea-
somal degradation. In contrast to the oxygen-dependent regu-
lation of HIF-1α degradation, we and others reported that growth
factor stimulation induces HIF-1α protein synthesis via a signal
transduction pathway leading from receptor tyrosine kinases to
PI3K to the serine/threonine kinases AKT and FRAP (mTOR; Refs. 29–34).
Up-regulated HIF-1α expression has been observed in
>70% of cancers including PCA as compared with adjacent
normal tissues (35, 36), and is likely achieved through both
epigenetic mechanisms (intratumoral hypoxia) and genetic al-
terations (mutations in tumor suppressor genes and oncogene
activation; Ref. 29). Overexpression of HIF-1α or HIF-1β-
dependent genes is associated with aggressive behavior in
human cancers in vitro as well as in clinical specimens (35, 37–45).
Hypoxic regions exist in human prostate carcinoma and
increasing levels of hypoxia are associated with higher clinical
stages (46). In mouse xenograft models, tumour growth and
angiogenesis are inhibited by small molecules and genetic stra-
tegies that disrupt HIF-1 activity but are stimulated by HIF-1α
overexpression (27, 47).
We tested the hypothesis that an antiangiogenic effect of
antiandrogens in androgen-responsive PCA cells can be regu-
lated by blocking HIF-1 transcriptional pathway. We found
that DHT stimulates HIF-1α protein expression, HIF-1 transcrip-
tional activity, and VEGF production in LNCaP cells, whereas
flutamide reduced these effects. Our experiments indicate that
androgenic induction of HIF-1α protein expression and function
are regulated in part through an autocrine loop mechanism
involving the PI3K/AKT pathway in PCA cells.

Materials and Methods

Cell Lines and Culture Conditions. The human PCA
cell lines LNCaP and PC-3 were purchased from American
Type Culture Collection (Manassas, VA), and were maintained
in RPMI 1640 supplemented with 10% fetal bovine serum at
37°C in a humidified atmosphere and 5% CO2 in air. The cells
were subjected to hypoxia in a sealed modular incubator cham-
ber (Billups-Rothenberg, Del Mar, CA) flushed with 1% O2, 5% 
CO2, and 94% N2 (1% O2), or to normoxia the cells were placed
directly in a 5% CO2 and 95% air incubator (20% O2), and
cultured at 37°C.

Reagents and Antibodies. DHT and flutamide were ob-
tained from Sigma-Aldrich (St. Louis, MO). CHX was from
BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA).
LY294002 was purchased from Alexis Biochemicals (San Di-
ego, CA). Human recombinant EGF was purchased from Life
Technologies, Inc. (Rockville, MD). R1881 was from Perkin-
Elmer, Inc. (Boston, MA). Bicalutamide (Casodex) was a gener-
ous gift from Dr. Leland W. Chung (Emory University, At-
lanta, GA). Purified mouse monoclonal anti-HIF-1α antibody
was obtained from BD Transduction Laboratories (Lexington,
KY). Polyclonal human antibody against HIF-2α was purchased
from Novus Biologicals (Littleton, CO). Antibodies against
human AR, actin, and VEGF (A-20) were from Santa Cruz
Biotechnology, Inc. (Santa Cruz, CA). Polyclonal human anti-
body to human TOPO-I was purchased from TopoGEN (Co-
lumbus, OH). The monoclonal antibody to rhEGF was pur-
bought from R & D Systems, Inc. (Minneapolis, MN). The
PhosphoPlus AKT (Ser473) Antibody kit for analysis of the
phosphorylation status of AKT and antibody to phospho-p44/42
MAPK (Thr202/Tyr204) were purchased from Cell Signaling
Technology, Inc. (Beverly, MA). Secondary antibodies were
horseradish peroxidase-conjugated and purchased from Amer-
sham Pharmacia Biotech (Piscataway, NJ).

Hormonal Treatment of LNCaP Cells. Only a low
passage number (up to 10) of LNCaP cells were used. Cells
were seeded in either six-well or 100-mm cell culture dishes and
grown in RPMI 1640 containing 10% fetal bovine serum until
50% confluence. The medium was then replaced with phenol-
red free RPMI 1640 containing 10% charcoal stripped serum
(androgen-free medium). After 24 h, the androgen-free medium
was refreshed and 1 nM DHT (dissolved in 100% ethanol), 1 μM
flutamide (dissolved in DMSO), or vehicle (0.1% ethanol and/or
0.1% DMSO) was added. The medium was changed at 2-day
intervals.

Protein Isolation and Western Blot Analysis. Cells
were washed twice with ice-cold PBS and then harvested,
scraped into ice-cold PBS, and pelleted by centrifugation at
500 × g for 5 min at 4°C. NE and CE were prepared as
described previously (18). Briefly, the packed cells were resus-
pended in 10 mM Tris HCl (pH 7.5), 1.5 mM MgCl2, and 10 mM
KCl freshly supplemented with 2 mM DTT, 0.4 mM phenylmeth-
ysulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml apportioning,
2 μg/ml pepstatin, and 1 mM Na3VO4. Cells were kept on ice
for 10 min. Nuclei were pelleted by centrifugation at 17,000
× g for 10 min at 4°C. The CE was kept, and the pellet was resuspended
in 0.5 M NaCl, 20 mM Tris HCl (pH 7.5), 20% glycerol, and 1.5
mM MgCl2 freshly supplemented with the protease and phos-
phatase inhibitors listed above, and then rotated for 30 min in
the cold room. The NE was cleared by centrifugation at
20,000 × g for 30 min at 4°C.

Proteins (30–60 μg/lane) from CEs or NEs were resolved
by 7.5% SDS-PAGE, electrotransferred to nitrocellulose mem-
brane, and incubated with the primary antibody. Immunoreac-
tivity was visualized by incubating the membrane with horse-
radish peroxidase-conjugated secondary antisera, followed by
the treatment with enhanced chemiluminescence reagent (Am-
ersham Biosciences, Piscataway, NJ). For detection with an-
other antibody, the membranes were stripped using a restore
Western blot stripping buffer (Pierce, Rockford, IL) and re-
probed with the desired antibody. TOPO-I and actin antibodies
were used as a loading control for NE and CE, respectively.

VEGF and PSA Measurement. LNCaP culture media
were collected, centrifuged to remove cellular debris, and stored
at −70°C until assayed for VEGF or PSA. VEGF assay was
performed using a commercially available ELISA kit (R & D
Systems, Inc.). PSA protein levels were determined using Micro- 
particle Enzyme Immunoassay (Abbott IMx PSA assay; Ab- 
bott Laboratories, Abbott Park, IL). Results between wells were 
standardized according to the amount of VEGF or PSA per total 
protein per well as measured in cell lysates and expressed as pg 
of VEGF protein per ml supernatant and ng of PSA protein per 
ml supernatant.

**Transient Transfections and Reporter Gene Assay.** 
LNCaP cells growing in six-well culture plates were transfected 
in triplicate with 1/2 H9262 g/well of reporter plasmid (pBI-GL V6L) 
containing HREs from the VEGF gene using GenePorter trans- 
fection reagent (Gene Therapy Sys, Inc., San Diego, CA) as 
described (49, 50). After 5 ho of transfection, the cells were 
allowed to recover overnight in androgen-free medium. The 
cells were then washed twice with PBS, and replenished with 
androgen-free medium and vehicle or reagent as indicated in the 
figure legends. Duplicate sets of transfected cell culture dishes 
were then separated and incubated either under normoxic or 
hypoxic conditions for 16 h. Luciferase activity was measured 
with commercial kit TROPIX (Bedford, MA) using a BMG 
Labtechnologies LUMIstar Galaxy luminometer and following 
the manufacturer’s instructions. Arbitrary Luciferase activity 
units were normalized to the amount of protein in each assay 
point. Protein concentration was determined using a BCA pro- 
tein assay kit (Pierce).

**Isolation and Analysis of RNA.** Total RNA was isolated 
using TRIzol Reagent (Life Technologies, Inc.) and was sub- 
jected (15 μg/sample) to Northern blotting using human HIF-1α 
cDNA probe (593-bp HindIII/MspI fragment) as described (51) 
or GAPDH and β-actin probes (Ambion, Inc., Austin, TX).

**Data Analysis.** Experiments presented in the figures are 
representative of three or more different repetitions. Quantifi- 
cation of band densities was performed using the public domain 
NIH Image (version 1.61). Statistical analysis was performed 
using a one-way ANOVA test (P < 0.05 was considered sta- 
tistically significant).

**Results**

**Androgens Induce Expression of HIF-1α Protein and 
HIF-1 Activity.** We first examined the effect of DHT and 
flutamide on HIF-1α and HIF-2α protein expression in LNCaP 
cells starting at 24 h after the culture medium was changed to 
androgen-free medium under normoxic conditions (Fig. 1A). 
Treatment with DHT showed an ~4-fold increase in HIF-1α 
after 24 h, which then increased to a maximum ~10-fold in- 
crease after 48 h. Additional incubation resulted in a gradual 
decrease in the levels of the protein. The time-dependent 
changes in expression of HIF-1α in LNCaP cells were similar to 
those of PSA (Fig. 1B) and VEGF (Fig. 1C) in the culture 
medium, but all were still higher in the presence of DHT than 
control and flutamide at each time point. VEGF protein expres- 
sion in cytoplasmic fraction was induced by DHT as well.

**Activation of AR stimulates HIF-1α expression in LNCaP 
cells. A, LNCaP cells were cultured in androgen-free medium for 24 h. 
They were then treated with vehicle (0.1% DMSO and 0.1% ethanol), 
1 nm DHT, or 1 μm flutamide under normoxic conditions for the 
indicated time (days). The cells were harvested, and NEs and CE 
s were prepared for Western blotting with antibody to HIF-1α and 
VEGF, respectively. The blots were stripped and reprobed with HIF-2α or 
TOPO-I and actin, respectively. The culture medium from the above 
incubations were analyzed for PSA (B) and VEGF proteins (C) as 
described under “Materials and Methods; bars, ±SD.
μM) inhibited the induction of HIF-1α protein by DHT (Fig. 2A). To measure the transcriptional activity of HIF-1, we used a reporter gene assay (Fig. 2B). LNCaP cells were transiently transfected with a construct containing Luciferase gene under the control of the HRE from the VEGF promoter (49). Consistent with the changes in HIF-1α protein levels, the hypoxia-induced HIF-1 transcriptional activation was enhanced 2-fold by DHT and was inhibited by flutamide (Fig. 2B). Because HIF-1α protein levels are very low in LNCaP cells under normoxia, HIF-1 transcriptional activity under normoxia measured by the reporter gene assay was undetectable (Fig. 2B). HIF-1α was also induced by low concentration (0.1 nM) of R1881, a nonmetabolizable synthetic androgen, and this induction was inhibited by the antiandrogen bicalutamide (Casodex; Fig. 2C).

**Androgen Induces HIF-1α through a Translation-dependent Pathway.** To better understand the processes involved in HIF-1α induction in response to androgen treatment, we investigated the effect of DHT and flutamide on the amount of HIF-1α mRNA. LNCaP cells were treated with DHT or flutamide, total RNA was extracted, and Northern blot analysis was performed using a HIF-1α cDNA probe (Fig. 3A). We found neither DHT nor flutamide treatment changed significantly HIF-1α mRNA levels, suggesting that androgens do not regulate HIF-1α mRNA transcription. In contrast, the amount of GAPDH mRNA, which is a HIF-1 regulated gene (52–54), was increased by DHT treatment suggesting the coactivation of HIF-1α transcriptional function in response to DHT (Fig. 3A). To analyze the effect of androgen on HIF-1α protein synthesis, we performed a time course of HIF-1α disappearance in the presence of the protein translation inhibitor, CHX (Fig. 3B). Within 15 min of CHX incubation HIF-1α protein fell to undetectable levels after DHT treatment. We compared the effect of DHT on HIF-1α with CoCl2 and EGF. CoCl2 mimic hypoxia and are known to block HIF-1α degradation but have no effect on HIF-1α synthesis (55). As expected in cells exposed to CoCl2, HIF-1α level remained almost stable for 60 min, despite the lack of ongoing protein synthesis (Fig. 3B). On the other hand, EGF has been shown in previous studies to regulate HIF-1α through PI3K/AKT pathway mainly by enhancing the efficiency of HIF-1α mRNA translation (29–31). Similar to EGF, the induction of HIF-1α by DHT was completely blocked by CHX within 15 min (Fig. 3C). Together, these results suggest that androgens increases HIF-1α levels through a translation-dependent pathway.

**Androgen Induces HIF-1α through an Autocrine PI3K/AKT-dependent Pathway.** We evaluated whether the effect of androgens on HIF-1α is mediated directly by the activation of the AR or is a consequent event of AR-downstream proliferative effects involving growth factors and their signaling pathways. To test that the effect of androgens on HIF-1α is mediated directly by the activation of the AR, we treated the hormone-insensitive PC-3 cells with DHT combined with bicalutamide at the indicated concentrations for 2 days under normoxic conditions. The cells were harvested, and NEs were prepared for Western blotting with antibodies to HIF-1α and TOPO-I.
Androgenic Regulation of HIF-1α

Androgen induces HIF-1α through a translation-dependent pathway. A, LNCaP cells were treated with vehicle, 1 nM DHT, or 1 μM flutamide for 2 days under normoxic conditions. The cells were harvested and total RNA was prepared for Northern blot analysis of HIF-1α, GAPDH, and β-actin. B, HIF-1α expression was induced by the exposure of LNCaP cells to DHT (1 nM) or CoCl2 (150 μM) under normoxia. After 4 h, CHX was added to a final concentration of 10 μg/ml, and the cells were harvested after being incubated for the indicated time in the presence of CHX and the inducer. Whole cell lysates were prepared and analyzed by Western blotting using antibodies to HIF-1α and actin. C, LNCaP cells were treated with vehicle, DHT (1 nM), or EGF (100 ng/ml) under normoxic conditions. After 48 h, 10 μg/ml CHX was added, and the cells were incubated in the presence of CHX for 15 min. The cells were then harvested and NEs were prepared for Western blotting using antibodies to HIF-1α and TOPO-I.

In Fig. 4B, the “conditioned” culture medium taken from LNCaP treated with DHT induced a modest increase in HIF-1α protein levels, whereas flutamide inhibited HIF-1α in ~70% compared with the control (Fig. 4B). This result suggests that conditioned medium contained soluble factors such as growth factors, secreted by LNCaP cells that affected HIF-1α levels in PC-3 cells. It seems that these “factors” were also present in the conditioned medium taken from control LNCaP cells, because HIF-1α levels were modestly enhanced by conditioned medium from DHT-treated cells but were inhibited by flutamide to an extent lesser than the control (Fig. 4B). The effect of conditioned medium from both untreated and DHT-treated LNCaP cells on HIF-1α was dose-dependent (Fig. 4C, top panel). Investigators have found that androgen stimulation of PCA cells in vitro leads to increased levels of secreted growth factors and their receptors including EGF (56, 57). On the basis of these reported findings, we anticipated that one of the soluble factors in LNCaP conditioned medium and that affects HIF-1α in PC-3 cells could be EGF. As depicted in Fig. 4C (bottom panel) HIF-1α levels in PC-3 cells incubated with conditioned medium from LNCaP cells treated with DHT were inhibited by increasing concentration of a blocking monoclonal antibody to rhEGF that neutralizes EGF bioactivity (R & D Systems, Inc.). This result suggests that a significant part of HIF-1α induction in PC-3 cells by LNCaP conditioned medium is derived from EGF activity. As a control for androgen response we measured VEGF (Fig. 4D) and PSA (Fig. 4E) levels in LNCaP conditioned medium used in PC-3 cells.

To test whether the up-regulation of HIF-1α by DHT is dependent on PI3K, we studied the effect of two inhibitors of tyrosine kinase receptor/PI3K, LY294002, and wortmannin in LNCaP cells. LY294002 completely blocked HIF-1α protein in DHT-treated LNCaP cells similar to those treated with EGF (Fig. 5A) as well as inhibited the DHT transactivation of HIF-1α-dependent reporter gene (Fig. 5B). Similar results were obtained by wortmannin (data not shown). Interestingly, flutamide failed to inhibit HIF-1α levels after EGF treatment (Fig. 5A) suggesting that the effect of AR activation on HIF-1α is upstream to EGF activity. To additionally confirm that the effect of LY294002 in LNCaP cells is because of PI3K pathway, we studied the phosphorylation status of AKT forms. As was shown previously, AKT was constitutively activated as a result of a frameshift mutation in the PTEN gene (58), but its phosphorylation was totally blocked by LY294002 in LNCaP cells in comparison with NIH-3T3 cells as a control (Fig. 5C, top panel). Neither DHT nor flutamide affected the activated AKT (Fig. 5C, bottom panel). On the other hand, EGF enhanced the activation of AKT, which was entirely blocked by the addition of LY294002 but not by flutamide (Fig. 5C, bottom panel). Furthermore, LY294002 blocked the phosphorylation of AKT in the presence of DHT, indicating that the inhibitory effect of LY294002 on DHT-induced HIF-1α is mediated through PI3K/AKT pathway. The effect of LY294002 was specific on PI3K because it had no effect on the EGF-activated forms of MAPK (Fig. 5D). In summary, DHT induces HIF-1α by increasing secretion of at least EGF, which through an autocrine mechanism activates PI3K/AKT pathway.

Discussion

The aim of this study was to test the hypothesis that HIF pathway is a direct downstream target of androgen action, and HIF-1α, HIF-2α, or both regulate androgen-induced VEGF expression in PCA cells.

Our results indicate that androgens and antiandrogens regulate HIF-1α expression, HIF-1 transcriptional activity leading to the secretion of VEGF (Figs. 1 and 2). HIF-2α was not affected by androgens or antiandrogens in the LNCaP system.
DHT and flutamide did not affect HIF-1α mRNA levels (Fig. 3A). Using the protein translation inhibitor CHX, we found that the induction of HIF-1α by DHT is likely dependent on a translation pathway rather than affecting the rate of HIF-1α protein stability or degradation (Fig. 3B). Given HIF-1α signaling by FRAP/mTOR (29–31), we suspect that DHT via FRAP/mTOR is increasing the rate of ribosomal recruitment to mRNA and, thus, enhancing synthesis of new HIF-1α protein molecules (27).

HIF-1 regulation was restricted only to androgen-
responsive LNCaP cells but not to the hormone-irrespective PC-3 cells. Interestingly, when PC-3 cells were treated with increasing amounts of conditioned medium from LNCaP cells, HIF-1α protein was induced in a dose-dependent manner (Fig. 4C). Most importantly, conditioned medium from LNCaP cells treated with flutamide inhibited HIF-1α expression compared with the other conditioned medium (Fig. 4B). This implied that the LNCaP cells secrete factors under the influence of the androgen and that these factors, rather than the androgen, resulted in the increased expression of HIF-1α. Because HIF-1α expression, particularly under normoxic conditions, is under the control of the receptor tyrosine kinase/PI3K/AKT signaling pathway (29–32), we anticipated that part of the factors effecting HIF-1α levels in the conditioned medium are likely growth factors such as EGF. Indeed, the stimulatory effect of the conditioned medium from LNCaP cells on HIF-1α in PC-3 cells was inhibited when conditioned medium were pretreated with antibody to rhEGF (Fig. 4B). These results suggest that an extracellular autocrine growth factor effect is involved in DHT stimulation of HIF-1α. Moreover, we observed that the DHT-induced HIF-1α and HIF-1 activity is totally inhibited by LY294002, whereas the EGF-induced HIF-1α was not affected by flutamide (Fig. 5). These responses were accordingly correlated with the phosphorylation status of AKT.

Hydroxyflutamide, an active metabolite of flutamide has been found previously to enhance AR translocation and to promote AR transcriptional activity in LNCaP cells as an AR agonist, because there is a mutation in the LNCaP AR gene (59, 60). As a positive control, we found that flutamide induced a small increase of PSA expression at 48-h time point, whereas DHT enhanced PSA expression by 3-fold (Fig. 1B). A similar dynamic pattern of expression was also observed on VEGF (Fig. 1C) and on HIF-1α (Fig. 1A) at the 48-h time point. Interestingly, when 1 μM flutamide was combined with 1 nM DHT, flutamide had an antagonistic effect on HIF-1α probably by competing with DHT as a weak agonist (Fig. 2). These effects are similar to what was reported previously with EGF; androgens induce a marked increase of extracellular EGF secretion, whereas this induction is blocked by the antiandrogen hydroxyflutamide in LNCaP cells (56). In addition to the increase in EGF release after androgen stimulation, both EGFR number and receptor binding affinity are also increased (57). Furthermore, EGF was reported recently to greatly enhance the expression of VEGF in androgen-independent PCA cell lines, PC-3 and DU-145 cells (61). Taken together, our results and findings pub-

Fig. 5  Androgens activate HIF-1 through a PI3K/AKT-dependent pathway. A, LNCaP cells were treated in the presence of the following additions: vehicle, 1 nM DHT, or 100 ng/ml EGF combined with 1 μM flutamide or 20 μM LY294002 as indicated on the top of each lane. NEs were prepared for Western blotting with anti-HIF-1α and anti-TOPO-I. B, LNCaP cells transiently transfected with pB-GL V6L were treated with vehicle or 1 nM DHT in the presence or absence of 20 μM LY294002 under hypoxic conditions. Luciferase reporter activity was then measured. Columns, means; bars, ±SD; n = 3; * P < 0.05. C, LNCaP cells were stimulated for 15 min with EGF (100 ng/ml) or DHT (1 nM) with or without pretreatment for 45 min with LY294002 (20 μM) or flutamide (1 μM) as indicated on the top of each lane. Whole cell extracts were prepared and analyzed by Western blotting using antibodies to phosphorylated AKT, phosphorylated p44/p42 MAPK, and total AKT. Whole cell extracts of NIH-3T3 cells untreated or treated with 50 ng/ml of platelet-derived growth factor (PDGF) were used as a control.

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lished previously lead us to propose a new model explaining the transcription factor cross-talk between AR and HIF-1 intracellular signaling pathways (Fig. 6). Androgen ligand would activate the AR and lead to proliferative effects on LNCaP cells, and consequently stimulates growth factor secretory responses including EGF secretion. Activation of growth factor receptors drives the synthesis of HIF-1α protein through PI3K/AKT/FRAP pathway, and then transcription of VEGF, survival genes, and so forth. ARE, androgen response elements.

The fact that the effect of androgens on HIF-1 activity is indirect and is regulated via a tyrosine kinase/PI3K pathway provides additional rationale for targeting HIF-1α or HIF-1 upstream pathways such as blockade of EGFR (65) especially in androgen-independent PCA. This is supported by data reported recently showing the antiangiogenic and antitumor activity of EGFR blockade either by the anti-EGFR antibody ImClone C225 (IMC-C225; Ref. 66) or the selective EGFR tyrosine kinase inhibitor ZD1839 (67).

Androgen regulation of HIF-1 occurs under normoxic conditions but can be additionally enhanced under hypoxia (Fig. 2B; Fig. 4D). This provides additional evidence that synergistic interactions exist between PI3K/AKT pathway activation and hypoxic pathway activation in PCA to regulate HIF-1α and drive angiogenesis (29). Antiandrogens are major antineoplastic drugs for advanced PCA. Our data suggest part of their clinical activity could involve reduction in HIF-1 transcriptional activation of VEGF and, thus, reducing angiogenic potential of androgen-sensitive clones.

Acknowledgments

We thank Dr. Jay N. Umbreit (Winship Cancer Institute, Department of Hematology and Oncology, Emory University, Atlanta, GA) and the honorable Hamilton Jordan (Georgia Cancer Coalition, Atlanta, GA) for critical discussions.

References


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