Testosterone-stimulated growth of the rat prostate may be driven by tissue hypoxia and hypoxia-inducible factor-1 α

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Abstract

Testosterone-stimulated growth of the ventral prostate (VP) in castrated rats is preceded by angiogenesis, but the mechanisms coordinating vascular and tissue growth are unknown. Adult rats were castrated and some treated with testosterone. Tissue hypoxia was studied morphologically using the hypoxia marker pimonidazole (Hypoxyprobe), hypoxia-inducible factor-1 (HIF-1) α , vascular endothelial growth factor (VEGF), and carbonicanhydrase 9 (CA-9) levels by western blotting and quantitative RT-PCR. In the intact untreated prostate, most glands were unstained by the hypoxia marker but already 1 day after castration most epithelial cells in the VP were stained. Seven days after castration prostate glands were apparently normoxic again, and HIF-1 α , VEGF, and CA-9 were decreased. Treatment of 7-day castrated rats with testosterone resulted in increased epithelial hypoxyprobe staining and increased HIF-1 α , VEGF,

and CA-9 levels. The transient increase in tissue hypoxia after testosterone treatment is probably caused by a temporary mismatch between oxygen consumption and supply. Treatment of prostate epithelial cells *in vitro* under normoxic conditions also increased HIF-1 α , and this could be blocked if epidermal growth factor receptor (EGFR) signaling was blocked with gefitinib. *In vivo* gefitinib could, however, not block the testosterone induced increase in HIF-1 α . Testosterone may thus induce HIF-1 α and its downstream angiogenesis promoting genes by at least two mechanisms, hypoxia and EGFR signaling. Transient epithelial cell hypoxia could by rapidly increasing HIF-1 α and VEGF be an essential coordinator of testosterone-stimulated vascular and glandular growth.

Journal of Endocrinology (2008) **196**, 11–19

Introduction

Androgens are the principal regulators of prostate growth and homeostasis. Androgen receptors are present on prostate epithelial and stroma cells, and androgens influence prostate growth by effects both in the epithelium and in the stroma (Isaacs *et al.* 1994, Kurita *et al.* 2001). We and others have shown that prostate blood vessels and prostate blood flow also play an important role in ventral prostate (VP) growth and regression (Lissbrant *et al.* 2001). Castration results in apoptotic death of most prostate epithelial cells and tissue involution, but this is preceded by a major decrease in blood flow (Lekås *et al.* 1997), endothelial cell death and vascular regression (Shabisgh *et al.* 1999, Johansson *et al.* 2005), and tissue hypoxia (Shabisgh *et al.* 2001). Insufficient blood flow and tissue hypoxia could actually trigger the subsequent death of epithelial cells (Lekås *et al.* 1997, 1999, Buttyan *et al.* 2000). Similarly, testosterone-stimulated growth of the VP in castrated animals is preceded by a major increase in blood flow, endothelial cell proliferation, and vascular growth (Lekås *et al.* 1997, Franck Lissbrant *et al.* 1998) suggesting that prostate growth is angiogenesis dependent (Folkman 1998, Franck Lissbrant *et al.* 1998).

As androgens regulate prostate growth and homeostasis partly by effects on the vasculature it is of interest to elucidate

mechanisms. Do androgens have direct effects on prostate blood vessels (Johansson *et al.* 2005) or are the effects mediated indirectly by effects on other prostate cells? And if so how? These questions are largely unanswered, but the observations that testosterone stimulates secretion of the potent angiogenic and vasodilatory factor, vascular endothelial growth factor (VEGF) from glandular epithelial cells in castrated animals (Joseph *et al.* 1997, Häggström *et al.* 1999), and that neutralization of VEGF bioactivity with a soluble VEGF receptor chimerical protein, mflt(1–3)IgG inhibits testosterone-stimulated prostate growth (Lissbrant *et al.* 2004) suggest that androgen receptor (AR)-positive epithelial cells may use VEGF as a signal to regulate the prostate vasculature. Tissue hypoxia generally increases the levels of hypoxia-inducible factor 1 α (HIF-1 α) and this is the major stimulator of VEGF, and other angiogenesis stimulators, secretion in various tissues (Dor *et al.* 2001, Giordano & Johnson 2001, Semensa 2003). It is therefore somewhat surprising that testosterone which increases blood flow in the VP (Lekås *et al.* 1997) also increases VEGF (Joseph *et al.* 1997, Häggström *et al.* 1999). In order to elucidate this apparent paradox further we studied tissue hypoxia, HIF-1 α , and VEGF in the rat prostate at different times after castration and testosterone treatment and found that testosterone treatment induces prostate tissue hypoxia and increases

HIF-1 α and that this may stimulate angiogenesis and coordinate this with prostate tissue growth.

Materials and Methods

Animals, treatments, and tissue hypoxia

The experimental design of this study proceeded according to the guidelines for care and management of laboratory animals and was approved by the local animal ethical committee. Animals were housed under a controlled temperature in an artificially illuminated room (12 h light/12 h darkness). Food and tap water were freely available. Adult male Sprague–Dawley rats (300–350 g, B&K, Stockholm, Sweden) were divided into several groups. Some rats were used as controls and others were castrated via the scrotum. Castrated rats were treated with testosterone esters (Sustanon, Organon, Oss, The Netherlands) given as a single subcutaneous injection of 10 mg/kg every morning as earlier described (Franck Lissbrant *et al.* 1998). Intact rats were also injected with 50 i.u. human chorionic gonadotropin (hCG; Organon, Oss, The Netherlands) to stimulate endogenous testosterone secretion and studied 4 and 24 h later. At different times after castration (1 day, 7 days) or after hCG treatment, the rats were injected intra-peritoneal with the morphological hypoxia marker pimonidazole hydrochloride, 60 mg/kg (Hypoxyprobe TM-1, Chemicon, Temecula, CA, USA, Raleigh *et al.* 2001). Similarly, 7-day castrated rats were treated with a single daily dose of testosterone and studied at different times thereafter (8 h, 1 day, 2, 3, and 4 days). Castrated and testosterone-treated rats were also treated with the tyrosine kinase inhibitor of EGF-R, gefitinib (150 mg/kg; donated by AstraZeneca, Södertälje, Sweden) as earlier described (Hammarsten *et al.* 2007). Pimonidazole is a low-molecular weight substance that after systemic injection from protein adducts in all cells in the body with a pO₂ of 10 mmHg or lower. Such adducts are then detectable with a specific monoclonal antibody (Hypoxyprobe TM-1Mab1, Natural Pharmacia International Inc., Belmont, MA, USA) following the manufacturer's instructions. One hour after intra-peritoneal injection of pimonidazole the rats were anesthetized and then fixed by vascular perfusion with buffered formalin, the prostate glands, seminal vesicles, the liver and the kidneys were removed, postfixed, dehydrated, and embedded in paraffin as earlier described (Franck Lissbrant *et al.* 1998). Four-micron thick sections were immunostained to detect tissue hypoxia following the manufacturer's instructions. Sections from the kidney and the liver were used as controls as these tissues normally contain parts with a pO₂ lower than 10 mmHg (Raleigh *et al.* 2001).

Other rats were treated as described but instead of perfusion fixation the tissues were removed and frozen in liquid nitrogen.

Cell line, culture conditions, and reagents

The human prostate epithelial cell line RWPE-1 was purchased from American Type Culture Collection (by way of LGC

Promochem, Borås, Sweden) and was maintained in Keratinocyte Serum-Free medium supplemented with 5 ng/ml human recombinant EGF and 0.05 mg/ml bovine pituitary extract (KSF medium, Invitrogen, formerly GIBCO-BRL) at 37 °C in a humidified atmosphere and 5% CO₂ in air.

R1881 and flutamide were obtained from Sigma–Aldrich. Cells were seeded in six-well culture dishes and grown in KSF medium until 50% confluence. The medium were then replaced with KSF medium without supplement. After 24 h, the medium was refreshed and 1 nM R1881 (dissolved in 100% ethanol), 1 or 10 μ M flutamide (dissolved in dimethyl sulfoxide (DMSO)), 100 ng/ml EGF, 1 or 10 μ M gefitinib (dissolved in DMSO), or vehicle (0.1% ethanol or 0.1% DMSO) were added and the cells were incubated for 2 days. Cells were washed with PBS and resuspended in cold lyses buffer A (see below).

To explore mechanisms behind pimonidazole staining RWPE-1 cells were grown in an incubator (Billups–Rothenberg, San Diego, CA, USA) at 21 or 1% O₂, at 37 °C for 2 h in KSF medium containing 100 μ M pimonidazole hydrochloride (Chou *et al.* 1996). Cells were washed with PBS to remove unbound pimonidazole, harvested by cytospin, fixed with acetone, and immunostained with an antibody for pimonidazole adducts as described above.

Protein extraction and Western blot

Frozen prostate tissues were pulverized in a micro-dismembrator (Braun, Biotech International, Sweden) and suspended in cold lyses buffer A (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), supplemented with complete protease inhibitors Boeringer Mannheim AB). Samples were incubated on ice for 20 min, 0.6% NP-40 was added, and cytoplasmic fraction (supernatants) were collected following refrigerated centrifugation. The resulting pellet was vigorously shaken, 4 °C, in buffer C (20 mM HEPES pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, and complete protease inhibitors) and nuclear extract was recovered following refrigerated centrifugation. Alternatively, whole-cell protein lysates were extracted in lyses buffer (7 M urea, 2 M thiourea, 2% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-1 propane sulfonate), 30 mM Tris pH 8.5, supplemented with complete protease inhibitors). Protein concentrations were determined with BCA Protein Assay kit (Pierce, Rockford, IL, USA) or 2D Quant kit (Amersham Bioscience). For Western blotting, protein samples were electrophoresed on 7.5% and 12% SDS-polyacrylamide gels under reducing conditions and fractionated proteins were electrophoretically transferred onto PVDF membranes (Amersham Bioscience). Membranes were blocked in 5% dry milk, 0.05% Tween-20 in PBS prior to incubation with mouse monoclonal antibody against human HIF-1 α (BD Transduction Laboratories, Stockholm, Sweden), rabbit polyclonal antibody against VEGF-A (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), rabbit polyclonal antibody against carbonic anhydrase 9 (CA-9, Novus Biologicals, Littleton, CO, USA), and rabbit polyclonal antibody against actin (Sigma–Aldrich).

After incubation with peroxidase-conjugated secondary antibodies (Amersham Bioscience) proteins were detected using enhanced chemiluminescence detection system (Amersham Biosciences).

HIF-1 α and VEGF-A mRNA expression

Total RNA was prepared from tissue samples using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA concentrations were determined spectrophotometrically at 260 nm (DU 640 Spectrophotometer, Beckman Coulter, Sweden) and RNA quality was evaluated electrophoretically (presence of 28/18S RNA). Five hundred nanograms of RNA were reversed transcribed with Superscript II reverse transcriptase (Invitrogen). Real-time PCR was performed using the LightCycler SYBR Green I technology (Roche Diagnostics). Reactions were performed in a 20 μ l volume with 0.5 μ M primers and 3 mM MgCl₂. Nucleotides, Taq DNA polymerase, and buffer were included in the LightCycler-FastStart DNA Master SYBR Green I mix (Roche Diagnostics). Primers were designed by the nucleotide sequence relative to VEGF-A GeneBank accession no M32977 (5'-TGCCAAGTGGTCCCAGGCTGC-3', 5'-CTCATCTCTCCTATGTGCTGGCC-3') and HIF-1 α GeneBank accession no NM024359 (5'-CCAAGGAGCCT-TAACC-3' and 5'-CCGTGCCCTACTATG-3'). The PCR was initiated with a 10-min enzyme activation step at 95 °C followed by 45 cycles of 95 °C denaturation for 15 s, 60 °C annealing for 10 s, and 72 °C extension for 10 s. To confirm amplification specificity, the PCR products were subjected to a melting curve analysis. Each experimental sample was run in duplicates and negative controls were always run in parallel. The quantification data were analyzed with the LightCycler analysis Software 3.5.3 (Roche Diagnostics). The relative values of the samples are calculated by a standard curve obtained by fivefold serial dilutions from a RNA sample in the intact group (double samples were run for each concentration). The same sample was used as calibrator for normalization between runs. The target values are presented as percent in comparison with the intact group.

Statistical analysis

Groups were compared using Mann–Whitney *U* test with five to nine animals in each group. Values are given as mean $\pm$ S.D., ^a*P* value <0.05 was considered significant.

Results

Castration and testosterone treatment induce transient tissue hypoxia in the ventral and dorsolateral prostate (DLP) lobes

In all animals, marked hypoxyprobe staining of similar intensity was observed in parts of the renal tubules and in the renal medulla, and in central parts of liver lobuli confirming that all animals had received the marker and

that it marked tissues known to be continuously hypoxic in normal rats (Fig. 1A, Arteel *et al.* 1995, Yin *et al.* 2002). Pimonidazole labeling does not detect minor or short-lived changes in hypoxia during a 1-h study period (Bennewith & Durand 2004).

In the VP lobe in intact untreated rats some glands, i.e. those with a highly active secretory epithelium showed staining whereas closely adjacent atrophic glands were unstained (Fig. 1B) demonstrating that tissue oxygen levels are related not only to supply but also to consumption. The glands in the DLP lobe were uniformly unstained (Fig. 1C). Twenty-four hours after castration all glands in the VP lobe were intensively stained (Fig. 1D), whereas glands in the DLP lobe showed weak staining for hypoxia or remained unstained (Fig. 1E). Seven days after castration the glands in the VP (Fig. 1F) and DLP (Fig. 1G) lobes were lined with an atrophic epithelium that was generally unstained by the hypoxyprobe suggesting a normoxic balance now based on low flow and low metabolism (Lekås *et al.* 1997).

Castrated rats were treated with a single daily dose of testosterone and tissue hypoxia was studied at different times thereafter. Eight hours after testosterone treatment a slight increase in hypoxyprobe staining was observed whereas treatment for 24 h caused a major increase in staining intensity in the VP lobe (Fig. 1H). In castrated rats treated with daily injections of testosterone for 2, 3, or 4 days, the hypoxyprobe staining gradually decreased from very intense levels seen at day 1 to values similar to that in intact animals seen at 4 days (Fig. 1J). Glands in the DLP were stained at 1 (Fig. 1I) and 2 days after testosterone treatment and thereafter showed weak staining for hypoxia or were unstained (Fig. 1K, 4 days of testosterone treatment). Treatment of intact rats with the luteinizing hormone agonist hCG for 24 h resulted in a markedly increased hypoxyprobe staining in the epithelial cells in the VP, demonstrating that also stimulation of the prostate gland with endogenously increased testosterone levels result in local tissue hypoxia (data not shown).

To test whether the hypoxyprobe staining in prostate epithelial cells were caused by a non-hypoxic mechanism that could metabolize pimonidazole, prostate epithelial cells were grown in a normoxic or a hypoxic environment. Cells grown under normoxic conditions were not stained whereas cells grown under hypoxia were stained (Fig. 2), suggesting that hypoxyprobe staining of prostate epithelial cells is caused by hypoxia and not by unspecific uptake.

Testosterone treatment increases HIF-1 α in the prostate

Expression of a 116 kDa protein referred to as HIF-1 α was detected in the VP and DLP lobes. Castration treatment caused slightly decreased levels of HIF-1 α protein in the VP lobe (Fig. 3A), but in the DLP HIF-1 α levels were increased 1 day after castration as described earlier (Fig. 3B, Shabsigh *et al.* 2001). Three days after castration HIF-1 α protein levels were decreased in both prostate lobes and 7 days after treatment HIF-1 α protein was almost absent, which is in line

Figure 1 Section from the kidney (A) and prostate glands (B–K) in rats injected with the hypoxia marker pimonidazole hydrochloride and immunostained to visualize hypoxic cells, 200× magnifications. The scale bars are 100 μm. Hypoxic red-brown stained cells were observed in some renal tubules in intact rats (A). Some cylindrical luminal epithelial cells in the ventral prostate (VP) in intact rats were stained by the hypoxia marker (arrowhead, B) but adjacent atrophic glandular cells were unstained (B). Hypoxic cells were not observed in the dorsolateral prostate (DLP) in intact rats (C). One day post-castration (d.p.c.) all glandular epithelial cells in the VP were stained by the hypoxia marker (D) and the DLP showed weak staining for hypoxia (E). Seven days post-castration the atrophic epithelial cells in the ventral prostate were unstained by the hypoxia marker (F) and the DLP showed weak staining (G). One day after testosterone treatment (and 8 days after castration, d.p.c. + T) all glandular epithelial cells in the VP were stained by the hypoxia marker (H). In the DLP, some hypoxic glands (arrowheads) were observed 1 day after testosterone treatment in castrated rats (I). After 4 days of testosterone treatment (and 11 days after castration), the glandular epithelial cells in the VP and DLP were only weakly stained by the hypoxia marker (J and K).

Figure 2 Prostate epithelial cells (RWPE-1) were incubated with pimonidazole hydrochloride and immunostained to visualize hypoxic cells (200 $\times$ magnifications). Cells grown in normoxia (21% O₂) did not stain (A) whereas cells grown in hypoxia (1% O₂, B) were intensely stained.

with the lack of hypoxyprobe staining at this time point (Fig. 3). Testosterone treatment of castrated animals resulted, within 2 days, in increased HIF-1 α mRNA (Table 1) and protein levels in the VP and DLP (Fig. 3).

Testosterone treatment affects HIF target gene expression

Two HIF target genes, VEGF-A and carbonic anhydrase 9 (CA-9), were detected in the rat VP and DLP (Fig. 3). VEGF-A mRNA expression in the VP was unaffected at one and three days, but decreased 7 days after castration (Table 1). The protein levels of VEGF-A in the VP were decreased at two days and undetectable 7 days after castration (Fig. 3A). In line with the hypoxyprobe data, castration did not affect VEGF mRNA and protein levels as drastically in the DLP as in the VP (Fig. 3B). Testosterone treatment, however, increased VEGF-A expression in both the VP and DLP in castrated rats (Table 1, Fig. 3). In line with VEGF castration decreased CA-9 protein expression 7 days after treatment in the VP but CA-9 protein

levels in the DLP were only slightly affected by castration (Fig. 3). CA-9 protein was increased in both the VP and DLP after testosterone treatment.

Androgens induce expression of HIF-1 α in prostate epithelial cells via EGF-R

Treatment with R1881, a non-metabolizable synthetic androgen, showed an increase in HIF-1 α expression after 2 days in RWPE-1 cells (Fig. 4). This induction was inhibited by the AR inhibitor flutamide demonstrating that androgens act through AR. Androgens have been shown to increase HIF-1 α in prostate cancer cells indirectly via EGFR (Mabjeesh *et al.* 2003). We therefore tested if androgens regulate HIF-1 α expression in non-malignant prostate epithelial cells in a similar way. Gefitinib (Iressa) an EGFR tyrosine kinase inhibitor decreased the induction of HIF-1 α in response to either EGF or R1881 stimulation in RWPE-1 cells (Fig. 4). To verify that gefitinib had the same effect also *in vivo*, 7 days castrated animals were treated with gefitinib or vehicle during testosterone stimulation. In this situation, gefitinib treatment had no effect on HIF-1 α protein expression, but led to a decrease in VEGF protein levels (Fig. 5) and prostate tissue growth (Hammarsten *et al.* 2007). Together these results indicate that androgens can induce HIF-1 α expression in prostate cells at least by two ways, either by a major stimulation of metabolism resulting in hypoxia or indirectly via the EGF receptor tyrosine kinase/PI3K/Akt signaling pathway.

Discussion

In the present study, we examined effects of castration and testosterone treatment on local tissue oxygen levels in the rat prostate using a morphological marker of tissue hypoxia (marks tissues with pO₂ level below 10 mmHg) and correlated this to changes in HIF-1 α and HIF target gene expression. The novel finding was that testosterone stimulation caused transient prostate epithelial cell hypoxia and that this may stimulate angiogenesis and coordinate vascular and glandular growth. Transient hypoxia could thus be of importance during prostate involution following androgen withdrawal (see introduction) and during testosterone-stimulated prostate growth.

The VP is known to be the most androgen-dependent part of the prostate gland in rodents, and this lobe is generally used in studies aiming to elucidate how testosterone influence prostate metabolism, cell proliferation, and cell death (Kurita *et al.* 2001). Castration reduces metabolism and consequently oxygen consumption in the prostate (Lee 1981), but also prostate blood flow (Lekås *et al.* 1997). Tissue hypoxia was observed 1–3 days after castration (Shabsigh *et al.* 2001), and not at 7 days (this study). Together, these observations suggest that castration only transiently disturbs the balance between local oxygen demand and supply. Early after castration, flow and oxygen supply apparently decreased more than metabolism resulting in transient tissue hypoxia. New normoxic equilibrium, with low metabolism and low flow, was in fact reached at 7 days after

Figure 3 Expression of HIF-1 α , VEGF-A, and CA-9 protein in the rat ventral (A) and dorsolateral (B) prostate after castration and following castration + testosterone stimulation for 1 day, 2, 3, and 4 days (as indicated). Each group was a pool of three to five rats in the Western blot procedure. Actin was used as a control for equal loading.

castration. In line with this, total VP protein synthesis is only slightly affected (Lee 1981), whereas flow is markedly reduced 24 h after castration (Lekås *et al.* 1997). At 7 days, both flow and metabolism are markedly reduced (Lee 1981, Lekås *et al.* 1997). The suggestion that tissue oxygen levels in the prostate are related both to oxygen supply and demand was also supported by the observation that localized tissue hypoxia could be seen in highly active secretory glands in the normal VP but not in closely adjacent glands lined with a more inactive atrophic epithelium glands all probably supported by the same vasculature.

In other tissues, local hypoxia is a major stimulator of VEGF (Dor *et al.* 2001, Giordano & Johnson 2001), but in the prostate the hypoxia response is inhibited in the absence of androgens (Stewart *et al.* 2001). This may explain why VEGF expression was not increased in the VP early after castration. This may also be true for expression of other HIF target genes like CA-9 in the prostate androgen free environment. Furthermore, ischemia

may play a slightly different role in different parts of the prostate; the DLP is less androgen sensitive than the VP (Banerjee *et al.* 1995). Glandular weight in the DLP is reduced after castration, but this is neither accompanied by a major apoptotic cell death in the epithelium (Banerjee *et al.* 1995, Kurita *et al.* 2001) nor by changes in blood flow (Lekås *et al.* 1997). In line with this, transient castration-induced tissue hypoxia and changes in the expression of HIF target genes in this study were not in the same magnitude in the DLP as in the VP.

Testosterone-stimulated growth of the VP and DLP in castrated rats is accompanied by vasodilatation, increased blood flow, endothelial cell proliferation and vascular growth, and increased epithelial cell VEGF synthesis (Joseph *et al.* 1997, Lekås *et al.* 1997, Franck Lissbrant *et al.* 1998, Häggström *et al.* 1999, Lissbrant *et al.* 2001). The testosterone-stimulated increase in endothelial cell proliferation and vascular growth and subsequent organ growth can

Table 1 Relative differences of hypoxia-inducible factor-1 α (HIF-1 α) and vascular endothelial growth factor-A (VEGF-A) mRNA in the rat ventral and dorsolateral prostate lobes at different times after castration and at different times after testosterone treatment ($n=5-9$ rats), for details see text

	Ventral prostate		Dorsolateral prostate	
	HIF-1 α (% $\pm$ s.d.)	VEGF-A (% $\pm$ s.d.)	HIF-1 α (% $\pm$ s.d.)	VEGF-A (% $\pm$ s.d.)
Intact	100 $\pm$ 44	100.0 $\pm$ 17	100 $\pm$ 72	100.0 $\pm$ 19
1 day castrated	116 $\pm$ 51	98.4 $\pm$ 11	123 $\pm$ 69	106.2 $\pm$ 30
3 days castrated	146 $\pm$ 48	100.3 $\pm$ 10	172 $\pm$ 117	NA
7 days castrated	104 $\pm$ 48	35.0 $\pm$ 28*	98 $\pm$ 56	79.2 $\pm$ 44
Castrated + testosterone 1 day	84 $\pm$ 21	63.0 $\pm$ 32 [†]	174 $\pm$ 152	160.2 $\pm$ 40* [†]
Castrated + testosterone 2 days	160 $\pm$ 44 [†]	102.8 $\pm$ 0.6 [†]	114 $\pm$ 50	89.5 $\pm$ 10
Castrated + testosterone 3 days	180 $\pm$ 77	105.9 $\pm$ 13 [†]	99 $\pm$ 48	NA

s.d., standard deviation; NA, not analyzed. *Significantly different than in intact rats, $P<0.05$, Mann-Whitney U test. [†]Significantly different than in 7-day castrated rats, $P<0.05$, Mann-Whitney U test.

Figure 4 R1881 (synthetic androgen) induces HIF-1 α protein in RWPE-1 cells. The androgen receptor inhibitor, flutamide, and EGF-R inhibitor, gefitinib, blocks HIF-1 α expression in response to R1881 or EGF. RWPE-1 cells were cultured in androgen-free medium for 24 h and then treated with vehicle, R1881, R1881 + flutamide, R1881 + gefitinib, EGF, EGF + gefitinib, flutamide, and gefitinib for 48 h. The experiment was repeated thrice with no apparent changes.

be inhibited by blocking VEGF (Lissbrant *et al.* 2004), demonstrating that testosterone-stimulated VP growth is angiogenesis dependent and that VEGF is a central mediator of this effect. Direct effects of androgens on prostate blood vessels are, however, also possible as mural cells in prostate blood vessels express AR (Johansson *et al.* 2005). In the present study, we demonstrate that testosterone treatment transiently induces tissue hypoxia in the VP and DLP, and suggest that this is caused by a temporary mismatch between a rapid major increase in metabolism and oxygen demands (Sensibar *et al.* 1990) and an apparently insufficient moderate increase in blood flow (Lekås *et al.* 1997). We also suggest that transient tissue hypoxia, by increasing local HIF-1 α and VEGF secretion and consequently vascular growth is an important component in androgen-stimulated prostate growth. Later during continuous testosterone stimulation when local oxygen demands and supply are balanced the tissue will again become normoxic, VEGF will normalize, and additional tissue growth will not take place (Sordello *et al.* 1998). Our hypothesis that testosterone-induced vascular and subsequent tissue growth in the prostate could be driven and coordinated by a temporary mismatch between local oxygen consumption and delivery is in line with the recent opinion that tissue growth and regression in general could be regulated by local tissue oxygen (Dor *et al.* 2001, Giordano & Johnson 2001, Lee *et al.* 2001). Embryonic development, tumor, and tissue growth require angiogenesis to provide blood flow to an increasing cellular mass. How the growing tissue senses the need for increased blood supply and how it signals this to the vasculature is beginning to be explored. Tissue oxygen is at the centre of a feedback between the vasculature and the tissue it serves (Dor *et al.* 2001). Tissue growth and/or increased metabolic activity cause increased local oxygen consumption. This leads to a moderate sub-pathological decrease in tissue oxygen, so-called local 'physiological hypoxia' (Dor *et al.* 2001). Hypoxia, by increasing HIF-1 α , directly stimulates secretion of angiogenic factors, principally VEGF-A (Semensa 2003). Interestingly, hypoxia may augment testosterone-stimulated prostate growth also by increasing the sensitivity of the AR to androgen stimulation

(Park *et al.* 2006). If local tissue hypoxia drives prostate growth in synergy with androgens this may explain previous observations of links between atherosclerosis, prostate hypoxia, and the pathogenesis of benign prostate hyperplasia and cancer. Atherosclerosis in the arteries supplying the prostate has been associated with an increase in the risk of benign prostate hyperplasia (Harvey 1995, Ghafar *et al.* 2002, Berger *et al.* 2006) and prostate cancer (Hager *et al.* 2006), and prostate cancer is associated with tissue hypoxia (Movsas *et al.* 2001) and increased HIF-1 α levels (Kimbrow & Simons 2006).

Prostate epithelial cell HIF-1 α expression is, apart from hypoxia, also stimulated by testosterone *in vitro* under normoxic conditions. This increase is blocked by flutamide showing that HIF pathway is a direct downstream target of androgen action. In addition, prostate epithelial cell VEGF secretion is stimulated by testosterone *in vitro* (Sordello *et al.* 1998), and HIF-1 regulates androgen-induced VEGF expression in prostate cancer cells (Mabjeesh *et al.* 2003). Several studies indicate that the effect of

Figure 5 Gefitinib treatment in 7 day castrated rats during testosterone stimulation had no effects on HIF-1 α protein expression in neither the ventral prostate (VP) nor the dorsolateral prostate (DLP). VEGF-A protein was down-regulated by gefitinib treatment during testosterone stimulation in the VP and to a lesser degree in the DLP compared with vehicle-treated + testosterone-stimulated rats. Each group includes three rats in the Western blot procedure. The relative index (RI) for VEGF-A was normalized against actin relative intensity.

androgen on HIF-1 α activity in prostate cancer cells *in vitro* is indirect and regulated via increased activity in the EGF receptor tyrosine kinase/PI3K/Akt signaling pathway (Zhong *et al.* 2000, Laughner *et al.* 2001, Mabjeesh *et al.* 2003). Moreover, EGF enhances the expression of VEGF in androgen-independent prostate cancer cells (Ravindranath *et al.* 2001). This study demonstrated that EGF treatment increased levels of HIF-1 α in non-malignant prostate epithelial cells and this was inhibited by gefitinib (Iressa). In line with earlier studies, gefitinib also inhibited androgen-stimulated normoxic expression of HIF-1 α in prostate epithelial cells *in vitro*. Together, this indicates that androgens act through the EGF receptor tyrosine kinase/PI3K/Akt signaling pathway. However, gefitinib did not prevent the testosterone-stimulated increase in HIF-1 α expression in castrated animals suggesting that the hypoxia induced increase in HIF-1 α is not dependent on increased EGFR signaling *in vivo*. In an earlier study by our group, this gefitinib treatment suppressed testosterone-stimulated EGFR phosphorylation and tissue growth *in vivo* (Hammarsten *et al.* 2007). Gefitinib + testosterone treatment *in vivo* did, however, reduce VEGF. This can possibly be explained by a direct effect on VEGF transcription via EGFR and phosphorylation of Sp-1 (Pore *et al.* 2006).

Conclusions

Testosterone-stimulated prostate growth in castrated rats is accompanied by transient hypoxia in prostate epithelial cells. This hypoxia may drive and coordinate vascular and tissue growth in the prostate by increasing the sensitivity to androgens (Park *et al.* 2006), and by increasing HIF-1 α and VEGF synthesis. In addition, testosterone may use an EGFR-dependent mechanism to increase HIF-1 α and VEGF synthesis also under normoxic situations.

Acknowledgements

Skilful technical assistance was given by Ms Sigrid Kilter, Ms Ulla-Stina Spetz, Ms Birgitta Ekblom, Ms Elisabeth Dahlberg, and Ms Pernilla Andersson. This study was supported by grants from the Swedish Cancer Foundation, the Swedish Research Council, the Lion's Cancer Research Foundation, Umeå University, Åke Wibergs Foundation, Sweden, and the University Hospital in Umeå. The authors declare that there is no conflict of interest that would prejudice the impartiality of this work.

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Received in final form 16 October 2007

Accepted 23 October 2007

Made available online as an Accepted Preprint
23 October 2007