Androgen Receptor Signaling: Mechanism of Interleukin-6 Inhibition

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ABSTRACT

Nonsteroidal signaling via the androgen receptor (AR) plays an important role in hormone-refractory prostate cancer. Previously, we have reported that the pleiotropic cytokine, interleukin (IL)-6, inhibited dihydrotestosterone-mediated expression of prostate-specific antigen in LNCaP cells (Jia et al., Mol Can Res 2003:1:385–92). In the present study, we explored the mechanisms involved in this inhibition and considered possible effects on AR nuclear translocation, recruitment of transcription cofactors, and the signaling pathways that may mediate this inhibitory effect. IL-6 neither induced nuclear localization of the AR nor inhibited dihydrotestosterone-induced nuclear translocation of the receptor. IL-6 did not affect AR or p160 coactivator recruitment to the transcription initiation complex on the prostate-specific antigen enhancer promoter. Moreover, it did not lead to the recruitment of the comediator silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) or histone deacetylase 1 (HDAC1) at the same sites. IL-6 did, however, prevent the recruitment of the secondary coactivator, p300, to the complex and partially inhibited histone H3 acetylation at the same loci. Furthermore, inhibition by IL-6 was not mediated by the mitogen-activated protein kinase or the Akt pathways and was partially abrogated by signal transducers and activators of transcription-3 knock-down using small interfering RNA. Our results show that IL-6 modulates androgen action through the differential recruitment of cofactors to target genes. These findings may account for the pleiotropic actions of IL-6 in malignant prostate cells.

INTRODUCTION

The only effective systematic treatment for prostate cancer is androgen ablation (1). Although the majority of prostate tumors regress after androgen ablation, disease progression inevitably recurs. Recent evidence suggests that growth of treatment-resistant tumors still depends on maintenance of androgen receptor (AR) signaling pathways (2–4). One mechanism proposed for the maintenance of AR signaling in treatment-resistant prostate tumors is nonsteroidal activation of the AR by cytokines [such as interleukin (IL)-6] and growth factors (such as epidermal growth factor) involving Ras/mitogen-activated protein kinases (MAPKs), Janus-activated kinase (JAK)/signal transducers and activators of transcription (STAT), and/or phosphatidylinositol 3’-kinase/Akt (5–12).

IL-6 is a pleiotropic cytokine produced by a number of cell types and is involved in the regulation of diverse physiological and pathological processes including hematopoiesis and inflammatory responses in target tissues. In prostate cancer, elevated IL-6 serum concentrations are associated with higher prostate-specific antigen (PSA) levels, tumor metastasis, and cancer morbidity (13). The LNCaP cell line, one of the most commonly used androgen-dependent prostate cancer cell models, expresses high-affinity receptors for IL-6 but does not secrete large amounts of IL-6 (14–17). The reported effects of exogenous IL-6 on this cell line are conflicting, with some studies showing growth stimulation (18, 19), and others showing growth inhibition (15, 20). It was recently proposed that binding of IL-6 to its receptor in LNCaP cells results in modulation of three major downstream signaling pathways, Akt, MAPK, and STAT3 (11). Whereas AR transactivation activity was enhanced by the STAT3 or MAPK pathways, activity was suppressed via the Akt pathway in that study. In other studies, IL-6 increased phosphorylation of MAPK and activated the AR possibly via phosphorylation at one of several putative phosphorylation sites (7, 8). The constitutive activation of the Ras/MAPK signaling pathway potentially reduces the androgen requirement of LNCaP cells for growth, PSA expression, and tumorigenicity (21, 22). The involvement of STAT3 after treatment of LNCaP cells with IL-6 is a result of JAK activation (7). STAT3 is a transcription factor that resides in the cytoplasm and most likely has been associated with oncogenesis because of its involvement in the regulation of cell cycle progression and apoptosis (23). A direct interaction between STAT3 and the AR has been proposed as a mechanism by which the JAK/STAT pathway modulates AR activity (7).

In our previous studies, using the endogenous chromatin-integrated PSA gene as a reporter of AR activity, we found that IL-6 inhibited dihydrotestosterone (DHT)-stimulated expression of PSA in LNCaP cells (24). To better understand the mechanism(s) of the IL-6 inhibitory action in LNCaP cells, in the present study we examined the nuclear translocation of the AR and coactivator/corepressor occupancy at different loci of the PSA gene and identified the key nonsteroidal signaling pathways modulating AR activity.

MATERIALS AND METHODS

Cell Culture and Materials. Human prostate cancer LNCaP cells obtained from the American Type Culture Collection (ATCC CRL-1740; Manassas, VA) were maintained in RPMI 1640 supplemented with 5% (v/v) fetal bovine serum and used between passages 25 and 50. DHT and trichostatin A (TSA) were purchased from Sigma Chemical Co. (St. Louis, MO), Recombinant human IL-6 was obtained from R&D Systems, Inc. (Minneapolis, MN). Bicalutamide was obtained from ICI Pharmaceuticals (predecessor of Astra–Zeneca, London, United Kingdom). Antibodies were anti-AR (N20), anti-SMRT, and anti-actin from Santa Cruz Biotechnology (Santa Cruz, CA); anti-Ach3, anti-p300, anti-HDAC1, and anti-p21 from Upstate Biotechnology, Inc. (Lake Placid, NY); anti-p44/42 MAPK, anti-phospho-p44/42 MAPK, anti-STAT3, anti-phospho-STAT3 (Tyr705), anti-Akt, and anti-phospho-Akt (Ser473) from Cell Signaling Technology, Inc. (Beverly, MA); and anti-PSA from DAKO Corp. (Carpinteria, CA). Anti-AR antibody (U402; sheep polyclonal antibody) was made in our laboratory. U0126 and LY294002 were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA). AG490 was obtained from Promega (Madison, WI). The chimeric AR-green fluorescent protein (GFP) plasmid (kindly provided by Dr Marco Marcelli; Baylor College of Medicine, Houston, TX) was generated by inserting the pcMV-AR vector into the multiple cloning site of the pEGFP-C1 (CLONTECH, Palo Alto, CA) using a KpnI site located 5’ of the ATG start codon and a BamHI site located at the 3’ end of the AR so that the GFP is located 5’ of the AR. The pEGFP-C1 encodes a red-shifted variant of wild-type GFP, which contains a double amino acid substitution of Phe46 to Leu and Ser47 to Thr.

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Immunofluorescence. LNCaP cells (4 × 10⁶ cells/well) were cultured into 8-well chamber slides (NucIon Lab-Tek II Chamber slide; RS Glass Slide, Naperville, IL) in 600 µl of phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped fetal bovine serum (CSS) and allowed to grow in steroid-free conditions for 3-5 days. The media were removed and replaced with 600 µl of the same media containing DHT (0-10 nM), IL-6 (10 ng/ml), or IL-6 and DHT in combination. After a 4-h treatment, cells were fixed for 10 min in 4% paraformaldehyde followed by fixation in methanol (−20°C) for 3 min and 1 min in acetone (−20°C) to permeabilize cells. To detect the endogenous AR, cells were incubated overnight at 4°C with affinity-purified sheep polyclonal antibody (U402) to the NH2-terminal sequence of AR (1:100 dilution) followed by incubation for 1 h with donkey antiserum immunoglobulins conjugated with Alexa 488 in the dark at room temperature. Negative controls included incubation with no primary antibody and blocking peptide. PC3 cells known to be negative for AR were used as a negative control. Specificity of the AR U402 sheep antibody was also confirmed by Western blotting in the presence and absence of blocking peptide. The slides were incubated overnight at 4°C with affinity-purified endogenous AR, cells were incubated overnight at 4°C for 3 days. Cells were then transfected with an AR-GFP plasmid using LipofectAMINE 2000 reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer’s instructions, with minor modifications. Transfection media were replaced after 20 h with 600 µl of phenol red-free RPMI 1640 containing 5% CSS with vehicle (ethanol), DHT (1 nm-10 nm), IL-6 (10 ng/ml), or IL-6 in combination with DHT. After a 24-h treatment, the media with fresh cytokine and/or ligand were replenished, and the cells incubated for an additional 4 h before fixation and confocal microscopy.

Confoal Microscopy and Imaging Analysis. The images of endogenous AR and transfected AR-GFP were produced using the Bio-Rad Radiance 2100 confocal microscopy (Bio-Rad Microscience Ltd., Hemel Hempstead, United Kingdom) equipped with three lasers [argon ion 488 nm (14 mW), Green HeNe 543 nm (1.5 mW), and Red Diode 637 nm (5 mW) outputs] and an Olympus IX70 inverted microscope. The ×40 water objective was used, and images were collected at zoom 2.0×. The Alexa 488 or GFP was excited with Ar 488 nm laser line, and the emission was viewed through a HQ515/30 nm narrow band barrier filter in photomultiplier 1. The density of endogenous AR in the cytoplasm of untreated and treated cells was measured using the LaserPix software program for the Microsoft Windows (Bio-Rad Microscience Ltd.). The expression of endogenous AR was examined in up to 60 cells in three separate experiments. The one-way ANOVA test and the Dunn’s test were used to detect significant difference between control and treatment groups. All analyses were performed using SPSS 11.0 for Windows Software (SPSS Inc., Chicago, IL). Statistical significance was accepted at P < 0.05.

Plasmid Transfection and Luciferase Detection. LNCaP cells (5 × 10⁴ cells/well) were plated in 96-well plates and grown in phenol red-free RPMI 1640 containing 5% CSS for 3 days. Cells were then transfected with an AR-responsive reporter, PSA-luc [100 ng/well; GGL3-PSA540-luc; provided by Bristol-Myers Squibb, (Princeton, NJ); Ref. 24], and pCMV-p300 [50 ng/well; provided by Dr. T-P. Yao (Duke University, Durham, NC)] or pCAT-basic (50 ng/well; Promega), a negative control, using LipofectAMINE 2000 (Invitrogen Corp.) according to the manufacturer’s protocol. After transfection, cells were grown in phenol red-free RPMI 1640 containing 5% CSS with DHT and/or IL-6 as indicated for 30 h. Luciferase assays were conducted as described previously (24). The results are given as fold activation and represent means ± SD of quadruplicate wells.

Small Interfering RNA (siRNA) Transfection. LNCaP cells (3 × 10⁵ cells/well) were plated in 6-well plates and grown in phenol red-free RPMI 1640 containing 5% CSS for 3 days. Cells were transfected with STAT3 siRNA or nonspecific siRNA (Dharmacon Inc., Lafayette, CO) at final concentration of 100 nM using Oligofectamine reagent (Invitrogen Corp.) according to the manufacturer’s instructions. After transfection, cells were grown in phenol red-free RPMI 1640 containing 5% CSS for 48 h and then treated with DHT and/or IL-6 as indicated for 18 h. Total RNA extraction and protein extraction were conducted respectively for further assessment.

Real-Time Reverse Transcription-PCR. Total cellular RNA was prepared and treated with RNase-free DNase I using SV Total RNA Isolation System (Promega). A two-step reverse transcription-PCR method was used using the TaqMan Gold reverse transcription-PCR kit (Applied Biosystems, Branchburg, NJ). The primers and probes were described previously (24). Triplicate PCR reactions were routinely conducted. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression was analyzed for each sample in parallel wells. The results are represented as PSA/GAPDH mRNA ratio.

Immunoblotting. After treatment with DHT and/or IL-6 as indicated, cells were harvested in 100 µl of radioimmunoprecipitation assay buffer (10 µM sodium phosphate (pH 7.2), 2 mM EDTA (pH 8.0), 150 mM NaCl, 50 mM NaF, 0.1% SDS, 1% IGEPL CA-630, 1% sodium deoxycholate, and 0.2 mM Na2VO4) that contained a mixture of mammalian protease inhibitors (Sigma Chemical Co.). Equal amounts of each extract were analyzed by SDS-PAGE. Proteins were transferred to Hybond-P membrane (Amersham Pharmacia Biotech, Piscataway, NJ) and probed with different antibodies as indicated. Horseradish peroxidase-conjugated antirabbit IgG antibody or horseradish peroxidase-conjugated anti-goat IgG antibody (Santa Cruz Biotechnology) was used as the secondary antibody. Detection was performed using the Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology) according to the manufacturer’s protocol. Chemiluminescence was analyzed using a Fluor-S Multi-Imager MAX instrument (Bio-Rad, Hercules, CA).

Chromatin Immunoprecipitation Assay. LNCaP cells (7 × 10⁶ cells/150-mm dish) were cultured in phenol red-free RPMI 1640 supplemented with 5% CSS for 3 days. Cells were treated with DHT and/or IL-6 for various times as indicated, and chromatin immunoprecipitation assays were conducted as described previously (24). Briefly, cells were cross-linked at room temperature for 10 min by using 1% formaldehyde. After sonication, the resulting soluble chromatin was diluted 1:10 with dilution buffer and immunoprecipitated by incubation with the indicated specific antibodies overnight at 4°C with rotation. The following day, chromatin-antibody complexes were isolated from solution by incubation with protein G-Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4°C with rotation. The Sepharose-bound immune complexes were washed as described previously. The immune complexes were eluted from beads with an elution buffer (1% SDS and 0.1 M NaHCO3) followed by DNA extraction. DNA samples from chromatin immunoprecipitation preparations were analyzed by real-time PCR using an iCycler optical system (Bio-Rad) and AmpliTaq Gold PCR master mix (Applied Biosystems). The primers and probes were as follows: enhancer forward (5'-GCCGT-GATCTGAGAGAGATATCATC-3') and reverse (5'-ACACCTTTTTTT-TCTTGATTGTTG-3'); E-P forward (5'-CAGTGCCCATGGTTTGT-TGGTT-3') and reverse (5'-AACCATACCACTCGATATTACAC-3'); enhancer probe (5'-CCCTAGATAGTCTCCATGAGTACCA-3') and reverse (5'-GG-GGGGAGGTACTGCTATG-3'); enhancer probe (5'-6FAM-TGCAAG-GTGCTGCTTTTCAAACATTCC-BHQ-1-3'); E-P probe (5'-6FAM-CCT- AACGCAATCTAACTAAAG-BHQ-1-3'); and promoter probe (5'-6FAM-CAATTACTGATACCCGTCAGTGAC-BHQ-1-3') (Biosearch Technologies, Novato, CA). Triplicate PCR reactions for each sample were conducted. The results are given as a percentage of input and represent mean values ± SD of triplicate determinations.

RESULTS

IL-6 Inhibits DHT-Mediated PSA Expression. We have reported previously that IL-6 inhibited DHT-mediated PSA expression at 10 nM DHT concentrations (24). The inhibition was also apparent at lower DHT concentrations (Fig. 1), indicating a general inhibitory mechanism across all tested concentrations of DHT, including expected castrate levels of the androgen.

IL-6 Does Not Affect the Distribution and DHT-Induced Nuclear Translocation of the AR. To investigate the possible mechanisms between IL-6 and AR activity, initially we evaluated the effect of IL-6 on AR subcellular localization using confocal microscopy. Although endogenous AR was observed in both the cytoplasm and nuclei in the absence of ligand, the majority was nuclear (Fig. 2A). Treatment with either 1 or 10 nM DHT decreased...
cytoplasmic AR levels to about 60% of untreated levels ($P < 0.0001$; Fig. 2B). The cellular distribution of AR in cells treated with IL-6 alone was not different from that seen in untreated controls. AR distribution in cells treated with DHT and IL-6 in combination was similar to that in cells treated with DHT alone (Fig. 2A and B). No AR immunostaining was observed in the negative controls. In parallel

experiments using a transiently expressed AR-GFP, the AR was both cytoplasmic and nuclear in untreated controls and in cells treated with IL-6 (10 ng/ml) alone. DHT (1 nM)-induced nuclear localization of the fusion protein was not prevented by combined treatment with IL-6 and DHT (Fig. 3).

IL-6 Interferes with DHT-Mediated Transient p300 Recruitment. In our experience, IL-6 consistently inhibits endogenous PSA gene expression in LNCaP cells (25). To understand the precise mechanism of this IL-6 inhibitory activity, we analyzed the AR occupancy, histone H3 acetylation, and cofactor recruitment on the PSA enhancer and promoter and a sequence between them (E-P; Fig. 4A) during the first 4 h of treatment with DHT or DHT + IL-6. IL-6 had little effect on DHT-mediated AR occupancy, which occurs predominantly on the enhancer, but significantly inhibited histone H3 acetylation, consistent with our previous findings (Ref. 25; Fig. 4B).

GRIP1, a member of the p160 family of coactivators, functions as a molecular adapter. Recruitment of GRIP1 to the PSA enhancer and promoter after treatment with DHT was not affected by IL-6 treatment. In contrast, the DHT-mediated transient recruitment of p300 on both the promoter and the enhancer was totally inhibited by IL-6 (Fig. 4B). The transient nature of p300 recruitment by DHT treatment was a constant finding in three independent experiments and was also reported by other researchers (26, 27). Neither AR, cofactor occupancy, nor histone H3 acetylation was observed in the E-P region. Neither silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) nor histone deacetylase 1 (HDAC1) was recruited to any of the three sites (Fig. 4B). In similar experiments, SRC1, RAC3, TAF II, NcoR, HDAC2, and HDAC3 also yielded negative results (data not shown).

We confirmed that SMRT and HDAC1 could be recruited to the PSA promoter and enhancer by treating the cells with the AR antagonist bicalutamide. Bicalutamide recruited AR to both the promoter and enhancer regions of the PSA gene, although the levels of AR occupancy are much lower than those obtained after DHT treatment (Fig. 5A). SMRT and HDAC1 were recruited to both loci under these conditions (Fig. 5A), indicating that the lack of SMRT/HDAC1 recruitment to these sites after stimulation with IL-6 was not due to failure of the chromatin immunoprecipitation assay. To further demonstrate that IL-6 inhibition of PSA expression is not related to HDAC recruitment, we examined DHT-mediated PSA expression after IL-6 treatment with or without TSA, a HDAC inhibitor. Although TSA increased DHT-mediated PSA expression by about 20% (data not shown).
shown), TSA had no significant effect on the IL-6-induced inhibition (Fig. 5B). In contrast, TSA treatment reversed the bicalutamide-induced inhibition of PSA expression by about 2-fold (Fig. 5C).

**Transient p300 Overexpression Does Not Abrogate IL-6 Inhibition of AR Transactivation Activity.** In an attempt to manipulate p300 activity, we cotransfected a mammalian expression vector of p300 along with a PSA promoter/enhancer-driven luciferase reporter into LNCaP cells that were subsequently treated with or without IL-6 and/or DHT (Fig. 6). IL-6 inhibited AR activity by 31%, and although p300 overexpression significantly stimulated the activity by more than 2-fold, IL-6 maintained an inhibition of 46%. This result is consistent with an inhibition of p300 recruitment to the PSA enhancer/promoter. On the other hand, p300 overexpression still resulted in increased luciferase activity even during IL-6 treatment. This finding indicates that the inhibition observed by IL-6 cannot be due solely to inhibition of all p300 functions in this overexpression system. The results from this experiment should be interpreted with caution because we assayed a transient reporter plasmid and not the endogenous gene.

![Fig. 4. Influence of interleukin (IL)-6 on dihydrotestosterone (DHT)-mediated transcription complex formation on the prostate-specific antigen gene enhancer and promoter. A, schematic representation of the prostate-specific antigen gene promoter and enhancer regions. Vertical dark bars, locations of AREs. Horizontal dark bars, real-time PCR targeted regions. E-P is a region between the enhancer and promoter without any known function. Arrow, transcription start site. B, LNCaP cells were incubated in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped fetal bovine serum for 3 days and then treated with DHT (10 nM) alone or in combination with IL-6 (10 ng/ml) for various times as indicated. Androgen receptor occupancy, histone H3 acetylation, and recruitment of cofactors were examined by chromatin immunoprecipitation analyses. p300 inset graphs, in a separate chromatin immunoprecipitation experiment, p300 recruitment on both enhancer (a) and promoter (b) were analyzed at more time points around the 1 h time point for both the DHT and DHT + IL-6 treatment regimens. Values are presented as percentage input and represent the mean values ± SD of triplicate real-time PCR assays. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were performed.

![Fig. 5. Bicalutamide and corepressor recruitment. A, LNCaP cells were incubated in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped fetal bovine serum for 3 days and then treated with bicalutamide (5 μM) for 1 h. Chromatin immunoprecipitation analyses of androgen receptor, SMRT, and HDAC1 on the prostate-specific antigen (PSA) gene promoter and enhancer were performed. Values are presented as described in Fig. 2A (except that the matched no antibody value was subtracted from each value). B, LNCaP cells were incubated in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped fetal bovine serum for 3 days and then treated with different combinations of dihydrotestosterone (10 nM), interleukin-6 (10 ng/ml), and trichostatin A (100 nM) for 18 h. PSA mRNA levels were measured by real-time reverse transcription-PCR. The PSA expression values are shown as PSA/GAPDH mRNA ratios. Values are presented as the means ± SD of triplicate real-time PCR assays. C, LNCaP cells were incubated in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped fetal bovine serum for 3 days and then treated with different combination of dihydrotestosterone (10 nM), interleukin-6 (10 ng/ml), and bicalutamide (5 μM) for 18 h. PSA mRNA levels were measured and analyzed as described in B. Results shown are representative of at least two independent experiments.
Inhibiting MAPK or Akt Pathways Does Not Restore IL-6-Induced Inhibition of PSA Expression. We used specific inhibitors to determine the relative contribution of the JAK/STAT3, MAPK, and phosphatidylinositol 3'-kinase/Akt signaling pathways to the IL-6 inhibitory activity on PSA expression (Fig. 7A). The efficacy of the inhibitors was evaluated by immunoblot analysis of phosphorylated target proteins. IL-6-induced p44/42 MAPK phosphorylation was totally inhibited by MAPK kinase inhibitor U0126 (Fig. 7B), but this did not influence DHT-dependent PSA expression or the inhibitory activity of IL-6 (Fig. 7D). Because Akt is constitutively active in LNCaP cells, phosphorylation of Akt was independent of IL-6 stimulation (Fig. 7C). In addition, the level of phosphorylated Akt remained unchanged in LNCaP cells cultured in RPMI 1640 without serum for 2 days (data not shown). Inhibition of the constitutively active Akt pathway by LY294002 (Fig. 7C) dramatically inhibited DHT-dependent PSA mRNA expression, indicating a significant involvement of this pathway in modulating AR activity. The magnitude of IL-6 inhibitory activity remained unaffected by LY294002 treatment (Fig. 7E).
IL-6 Inhibition of PSA Expression Is Partially Abrogated after Knock-Down of STAT3. Although STAT3 phosphorylation was stimulated by IL-6 treatment (Fig. 8A), we were unable to inhibit STAT3 phosphorylation with the putative JAK inhibitor AG490. Increasing the concentration of AG490 to 100 μM (10× the IC50) and extending pretreatment time to 16 h did not inhibit phosphorylation of STAT3 but instead induced cytotoxicity (data not shown). Therefore, we used an alternative approach of knocking-down STAT3 expression with siRNA.

After transfection of LNCaP cells with STAT3 siRNA, total endogenous STAT3 levels were reduced by 70–80% (range of inhibition in three separate experiments as assessed by Western quantitation; Fig. 8B). IL-6-induced phosphorylation of STAT3 was also inhibited to a similar extent (about 70%) by STAT3 siRNA transfection (Fig. 8C). DHT-mediated and IL-6-inhibited PSA expression levels were reduced by the transfection of scrambled, control siRNA compared with untreated or mock-transfected controls, indicating a general siRNA effect on the cells (Fig. 8D). The inhibition by IL-6 remained at about 50% under these conditions. Nevertheless, the specific knock-down of STAT3 increased DHT-mediated and partially abrogated the IL-6 inhibitory effects on PSA mRNA and protein levels (Figs. 8, D and E, respectively). GAPDH mRNA levels remained essentially unchanged by all of the above-mentioned treatments. In the same experiment, knock-down of STAT3 did not significantly affect endogenous AR and p21 levels (Fig. 8E).

DISCUSSION

In prostate cancer, elevated IL-6 levels have been correlated with metastasis, morbidity, and poor prognosis (17, 28, 29). However, it is not clear how IL-6 signaling is involved in prostate cancer progression because previously published data have yielded conflicting results regarding the effects of IL-6 on prostate cancer cell proliferation (15, 18–20). We reported previously that in early- passage LNCaP cells, IL-6 inhibited DHT-stimulated cell growth and PSA gene expression (24). To gain insight into possible mechanisms involved in this inhibition, we considered, in the present study, nuclear translocation of the AR, recruitment of cofactors to the PSA enhancer and promoter, and, finally, which signaling pathway may be involved between the cell surface binding of IL-6 and DHT-mediated PSA expression.

In the present study, we observed persistent nuclear expression of endogenous AR after 3–5 days of culture in steroid-free medium. Similarly, nuclear AR was also detected by Lin et al. (30) in studies using LNCaP. However, contrary to the latter report, we did not observe increases in nuclear AR levels after IL-6 treatment. Cell-specific factors are known to alter AR nuclear trafficking (31) and may account for the differences between our observations and those of others.

It is well established that AR activates gene transcription by binding to specific androgen response elements on androgen-regulated target genes and recruiting coactivators in a ligand-dependent manner starting with the p160 family members, followed by p300 complexes (26), p300 recruits additional factors with histone acetyltransferase (HAT) activity, such as p300/CREB-binding protein (CBF)-associated factor, leading to histone acetylation, chromatin modification, and transcription initiation. Here, we found that IL-6 inhibited histone acetylation by interference with the DHT-induced transient p300 recruitment to p160 at the PSA enhancer and promoter. The reason of the transient nature of p300 recruitment is unknown. It might be an artifact of epitope masking, or it could be due to its replacement by other cofactors with additional HAT activities for further maintenance of histone acetylation. In this regard, our results are directly opposite
to that reported previously (32). The reason for this is unknown but might be related to the passage number of LNCaP cells used; we routinely use LNCaP cells at a passage number as low as possible and never beyond 50 passages. We also demonstrated that the mechanism of IL-6 inhibitory activity on PSA expression was different from that of the AR antagonist bicalutamide, which binds AR and recruits corepressors and HDAC (26, 33).

It is interesting to note that although AR recruitment levels to the promoter of the PSA gene were much lower than those to the enhancer, p160 and p300 recruitment to both loci were nearly equivalent. The reason for the lower AR occupancy at the promoter might be related to the fewer response elements at the promoter compared with the enhancer, but the high p160/p300 occupancy at the promoter might be the result of bridging activity of the cofactors between the two sites after DNA looping. It is known, for example, that p160 cofactors have more than one AR binding site (34) that might facilitate such bridging.

Emerging evidence indicates that coactivators and corepressors are themselves targets of multiple signal transduction pathways (35). Both p160 cofactors (36) and p300 (37) can be modulated by the action of several kinases. Activation of various intracellular signaling pathways and kinase cascades by membrane receptors modulates coactivator complexes and seems to determine which acetyltransferases, such as p300/CBP or p300/CBP-associated factor, are recruited to a particular coactivator complex in a specific context. Although the transient nature of p300 recruitment after treatment with DHT remains unexplained, a similar observation was made for estrogen receptor-regulated transcription complexes in which the transient recruitment of p300 was replaced by CBP (38).

In a recent report, IL-6 was shown to enhance AR transactivation via either STAT3 or MAPK pathways and suppress AR transactivation via the phosphatidylinositol 3’-kinase/Akt pathway (11). We found, however, that the Akt pathway might be a major constitutive contributor to nonsteroidal AR activation in LNCaP cells. Blocking this pathway significantly reduced DHT-mediated PSA expression without affecting inhibition of IL-6. In our experiments, IL-6 inhibited PSA gene expression, at least in part, through the STAT3 pathway without MAPK involvement, which is consistent with a report that the activation of STAT3 is the main underlying mechanism for IL-6-induced growth inhibition (39). This inhibitory activity did not result from modulation of endogenous AR and seems to be gene specific because we showed that AR and p21 protein levels remained unchanged. We speculate that activation of STAT3 would block the association between p160 and p300 on the PSA gene enhancer and promoter, thereby releasing HAT and preventing histone acetylation.

It remains unknown how activation of STAT3 may interact with AR and/or specific cofactors to influence the recruitment of p300 to transcription initiation complexes on the PSA gene promoter and enhancer regions. Because we did not find STAT3 occupancy on either the promoter or enhancer of PSA gene (data not shown), it remains possible that the JAK/STAT3 pathway activated a transcriptional repressor (such as cyclin D1) that inhibits PSA expression. Cyclin D1 can bind the amino-terminal domain (NTD) of the AR independent of its LXXLL motif and functions as a corepressor to inhibit ligand-dependent AR activation. Because cyclin D1-mediated repression is dominant over both HAT and HAT recruiting coactivators (40), it has been suggested recently that cyclin D1 binding to the AR may repress ligand-dependent AR activity by directly competing for p300/CBP-associated factor binding (41). Moreover, protein inhibitor of activated STAT3 (PIAS3) may also play a role in this process; PIAS3, originally identified as a specific inhibitor of STAT3, was recently shown to interact with the AR and suppress AR-mediated gene activation (42, 43). It should be noted that the transcription of control, scrambled siRNA resulted in a significant inhibition of PSA mRNA expression (under both DHT-treated and DHT+IL-6-treated conditions). This inhibition could possibly be due to general effects of duplex RNA species on the IFN-mediated activation of JAK/STAT pathways (44). If this is true, our results are consistent with an inhibitory activity of STAT3 on DHT-mediated PSA expression, which was rescued by the specific knock-down of STAT3.

Whereas the pleiotropic effects of IL-6 on the LNCaP phenotype, as reported by different groups, are puzzling, the challenge is to sort out how and by what mechanisms different signaling pathways affect AR-mediated gene expression. A better understanding of the diverse mechanisms involved in the regulation of AR signaling is essential to better control the growth of prostate cancer cells and, in particular, prevent the development of hormone-refractory prostate cancer, which currently is not amenable to alternative treatments. Conceivably, differential regulation of AR activity by multiple signaling pathways, including IL-6, could significantly affect the phenotype of prostate cancer cells and contribute to uncontrolled growth. Molecular detail of nonsteroidal modulation of the AR in the future might lead to cytokine-mediated control of prostate cancer cell growth, especially in a castrate environment.

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