

Interleukin-6 Induces Androgen Responsiveness in Prostate Cancer Cells through Up-Regulation of Androgen Receptor Expression¹

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ABSTRACT

Interleukin-6 (IL-6) induces prostate cancer (CaP) cell proliferation *in vitro*. Several lines of evidence suggest that IL-6 may promote CaP progression through induction of an androgen response. In this work, we explored whether IL-6 induces androgen responsiveness through modulation of androgen receptor (AR) expression. We found that in the absence of androgen, IL-6 increased prostate-specific antigen (PSA) mRNA levels and activated several androgen-responsive promoters, but not the non-androgen responsive promoters in LNCaP cells. Bicalutamide, an antiandrogen, abolished the IL-6 effect and IL-6 could not activate the PSA and murine mammary tumor virus reporters in AR-negative DU-145 and PC3 cells. These data indicate the IL-6 induces an androgen response in CaP cells through the AR. Pretreatment of LNCaP cells with SB202190, PD98059, or tyrphostin AG879 [p38 mitogen-activated protein kinase (MAPK), MAP/extracellular signal-regulated protein kinase kinase 1/2, and ErbB2 MAPK inhibitors, respectively) but not wortmannin (PI3-kinase inhibitor) blocked IL-6-mediated induction of the PSA promoter, which demonstrates that IL-6 activity is dependent on a MAPK pathway. Finally, IL-6 activated the AR gene promoter, resulting in increased AR mRNA and protein levels in LNCaP cells. These results demonstrate that IL-6 induces AR expression and are the first report of cytokine-mediated induction of the AR promoter. Taken together, our results suggest that IL-6 induces AR activity through both increasing AR gene expression and activating the AR in the absence of androgen in CaP cells.

These results provide a mechanism through which IL-6 may contribute to the development of androgen-independent CaP.

INTRODUCTION

When initially identified, most CaP³ require androgenic stimulation for growth. After androgen withdrawal, most prostate cells undergo an active process of programmed cell death (1). Unfortunately, after an initial response to androgen deprivation therapy, CaP usually recurs in a form that grows independent of androgen and is unresponsive to further androgen withdrawal (2). The mechanism responsible for development of androgen independent cancer is unknown. However, accumulating evidence suggests that the androgen-independent phenotype results when CaP cells acquire a paracrine or autocrine growth mechanism through production of growth factors and cytokines (3–5).

A putative growth factor that promotes prostate cancer growth is IL-6. Initially identified as a cytokine that exhibits pleiotropic functions, IL-6 regulates gene expression in a number of different organs, modulates immune function, stimulates the hypothalamic-pituitary axis, promotes osteoclast resorption of bone, and stimulates bone marrow (reviewed in Ref. 6). It has been shown to induce acute-phase proteins and a number of immediate early genes, including *jun B*, circulating intercellular adhesion molecule 1 (*ICAM-1*), and IFN regulatory factor 1 (*IRF1*; Refs. 7–9). The biological activities of IL-6 are mediated by the IL-6 receptor, which binds IL-6 specifically and with low affinity, and gp130, which associates with the IL-6-IL-6 receptor complex, resulting in high-affinity binding and activation of intracellular signaling. Evidence has accumulated that suggests IL-6 may be an important autocrine and/or paracrine growth factor for CaP (4, 5, 10, 11).

AR, an essential mediator of androgen action, is a ligand-dependent transcription factor belonging to the nuclear steroid hormone receptor superfamily (12). The receptor contains a ligand (androgen)-binding domain and a DNA-binding domain. In the absence of androgens, the AR stays mainly in the cytoplasm in an inactive form. When the AR is activated by androgen, it binds to an enhancer ARE in the regulatory region of target genes as the key step for promoter activation (13). During the progression of CaP, AR expression becomes heterogeneous (14).

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³ The abbreviations used are: CaP, prostate carcinoma(s); AR, androgen receptor; ARE, androgen responsive element; BIC, bicalutamide; DHT, dihydrotestosterone; ER, estrogen receptor; IL-6, interleukin-6; lux, luciferase; MMTV, murine mammary tumor virus; NFD, nonfat dried milk; PSA, prostate-specific antigen; RT-PCR, reverse transcription-PCR; SLP, sex-limited protein; PI, phosphatidylinositol; MAP, mitogen-activated protein; MAPK, MAP kinase; MEK, MAPK kinase; EGF, epidermal growth factor; GFP, green fluorescent protein; STAT, signal transducer and activator of transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

LNCaP is an androgen-responsive human CaP cell line, derived from a lymph node CaP metastasis (15). LNCaP cells have AR mutations and have been shown to be sensitive to antiandrogens and estrogen as well as to androgens (16, 17). IL-6 has been shown to confer androgen-like activity on LNCaP cells (18, 19). Although the ability of IL-6 to induced androgen-like activity has been associated with activation of several signal transduction cascades (19, 20), the mechanism through which IL-6 induces AR activity is unknown. Accordingly, in the current report, we explore the mechanism through which IL-6 induces an androgen response in prostate cancer cells.

MATERIALS AND METHODS

Cell Lines and Reagents. LNCaP, PC-3, and DU-145 prostate cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD). These cell lines have been previously shown by several investigators to express both the IL-6 receptor α and the IL-6 receptor β (gp130) chains (4, 5, 21). The cells were maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in a 5% CO₂ incubator. Recombinant human IL-6 was obtained from Sigma Chemical Co., Inc. (St. Louis, MO). Rabbit antihuman AR polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz Biotechnology, CA). The antiandrogen BIC (Casodex, 20 μ M; Zeneca Pharmaceuticals; Macclesfield, United Kingdom) was provided by Dr. K. Olsen (University of Michigan, Ann Arbor, MI). The following reagents: BSB202190 (5 μ M), PD98059 (15 μ M), wortmannin (100 nM), and tyrphostin AG879 (10 μ M) were purchased from Calbiochem, San Diego, CA. The PSA reporter, p1.5kPSA-Lux, containing the proximal -1500-bp of the PSA promoter driving the lux cDNA was a gift from Dr. P. Hsiao (NIEAS, Durham, NC). The MMTV (22) reporter, pMMTV-Lux, containing 150 bp of the MMTV promoter driving the lux cDNA has been described previously (23). The mouse SLP (24) reporter, p Δ 9(slp)-Lux, containing 900 bp of the SLP promoter driving the lux cDNA is a gift from Dr. D. Robins (University of Michigan, Ann Arbor). The plasmid pPAI(-800/+22)-Lux containing a human type-1 plasminogen activator inhibitor gene promoter driving the lux cDNA was a gift from Dr. T. Gelehrter (University of Michigan, Ann Arbor). The plasmid ERE-tk-Lux, containing tandem ER response elements upstream of the thymidine kinase promoter driving the lux cDNA, was a gift from Dr. R. Koenig (University of Michigan, Ann Arbor).

Semiquantitative RT-PCR. Semiquantitative RT-PCR was performed as previously described, with minor modification (18). Briefly, total RNA was isolated from cells using TRIzol method (Life Technologies, Inc.). Using Superscript one-step RT-PCR system (Life Technologies, Inc.), we subjected 1 μ g of total RNA to thermal cycling as follows: one cycle at 50°C for 30 min; at 94°C for 2 min with an additional 29 cycles at 94°C for 15 s; at 56°C for 30 s; and at 72°C for 1 min, and 5 min at 72°C for the final extension. PSA and β 2-microglobulin primer sequences were: PSA 418/21 sense, 5'-GGCAGGTGCTTG-TAGCCTCTC-3'; PSA 939/21 antisense, 5'-CACCCGAGCAG-GTGCTTTTGC-3'; β 2-microglobulin, sense, 5'-ATGCCTGC-

CGTGTGAACCATGT-3'; and β 2-microglobulin antisense 5'-AGAGCTACCTGTGGAGCAACCT-3' as previously described (18). The AR primers were designed to flank the ligand-binding domain: sense, 5'-ACACATTGAAGGCTATGAATGTC-3'; and antisense, 5'-TCACTGGGTGTGGAAATAGATGGG-3'. For quantitation, either PSA or AR primers (10 μ M, 1 μ l) was mixed with β 2-microglobulin (5 μ M, 1 μ l) for RT-PCR. The PCR products were then resolved in the 1.3% agarose gel and bands were analyzed with ChemiImager v3.3 software (Alpha Innotech, San Leandro, CA). Target fragment levels were normalized against β 2-microglobulin, and data are presented as target mRNA: β 2-microglobulin ratio.

Transient Transfection. Transfection was conducted using SuperFect (Qiagen, Valencia, CA) as recommended by the manufacturer. Briefly, LNCaP cells were plated in 6-well plates at a confluency of 60–70% 24 h before transfection in medium supplemented with fetal bovine serum or in medium supplemented with charcoal-stripped serum as indicated (See Fig. 1). Cells in 1 ml of medium in 6-well plates was incubated at 37°C with 2 μ g of plasmid DNA (1 μ g of reporter, internal control 50 ng of pRL-cytomegalovirus, and 950 ng pBluescript) mixed in 10 μ l of SuperFect. After the 2 h incubation, 1 ml of fresh medium was added to the cells. The medium was replaced the next day, and the cells were incubated for an additional 24 h. Total protein was then collected by lysis buffer and lux activities were measured by using Dual-Luciferase System (Promega, Madison, WI) and captured by TD-20/20 Luminometer (Turner Designs, Sunnyvale, CA).

Nuclear Lysate Preparation. Nuclear protein extract from LNCaP cells was prepared as described previously (23). Briefly, cells were harvested after being washed twice in PBS buffer. For nuclei preparation, cells were resuspended in hypotonic buffer [10 mM HEPES-KOH (pH 7.9), 1.5 mM MgCl₂, 10 mM KCl, and 0.1% NP40] and incubated on ice for 10 min. Nuclei were precipitated with 3000 \times g centrifugation at 4°C for 10 min. After washing once with hypotonic buffer, the nuclei were lysed in the lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Triton X-100) and incubated on ice for 30 min. The nuclear lysates were precleared by 20,000 \times g centrifugation at 4°C for 15 min. Protein concentration was measured by Bradford assays.

Western Blot Analysis. LNCaP cells were cultured for 2 days in media with charcoal stripped serum. The cells were then stimulated for 24 h with the indicated concentrations of IL-6 and then lysed by multiple freeze thawing in 0.25 M Tris buffer. Western blot analysis was performed as previously described (23) with rabbit anti-AR (N-20, Santa Cruz Biotechnology).

Detection of AR by Indirect Immunofluorescence and Fluorescence Imaging. LNCaP cells were grown on glass coverslips for immunofluorescence. The coverslips were rinse once in PBS and fixed with 4% paraformaldehyde in PBS pH 7.4 for 10 min. Neutralization took place for 5 min in 50 mM NH₄Cl in PBS. The coverslips were then washed twice in PBS. Cells were incubated for 15 min with 1% BSA, NFD, 0.3% Triton X-100 in PBS. The coverslips were incubated for 1 h in rabbit anti-AR (N-20) diluted 1:100 in 1% BSA, 5% NFD, and 0.1% Tween 20 in PBS. The slips were then extensively washed in PBS-Tween-20 and incubated for 1 h with goat antirabbit IgG conjugated with FITC (Santa Cruz Biotechnolo-

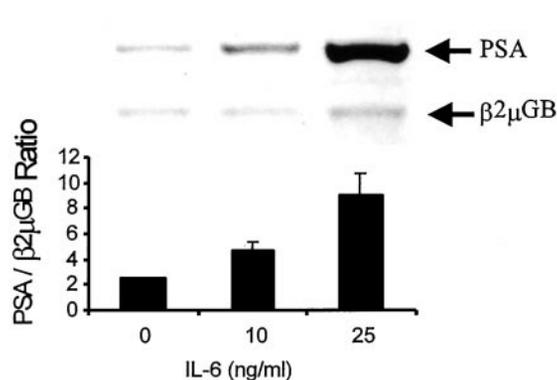


Fig. 1 IL-6 induces PSA mRNA levels from LNCaP cells in the absence of androgen. LNCaP cells were plated to a 60% confluence in charcoal-stripped media in 100-mm plates. Cells were then treated with 0, 10, or 25 ng/ml IL-6 for 24 h. After incubation, total RNA was collected and evaluated for the PSA mRNA levels by semiquantitative RT-PCR. PCR products were subjected to electrophoresis and measured by densitometry. The mRNA levels are expressed as the ratio of PSA: β 2-microglobulin. The gel shown here is the result of a typical experiment. Results are shown as mean (\pm SE) from three independent experiments.

gy), diluted 1:500 in 1% BSA, 5% NFD, and 0.1% Tween 20 in PBS. The images were captured using a fluorescent microscope. A GFP-AR expression plasmid (a gift from Dr. M. Lu, Harvard University, Boston, MA) was transfected into PC3 cells. The fusion fluorescent protein was imaged by confocal microscopy.

RESULTS

IL-6 modulates both cell growth (5, 21, 25, 26) and the androgen regulatory signal pathway in LNCaP cells (18, 19). However, results regarding IL-6 expression and activity in LNCaP cells are rather inconsistent (27). To determine whether IL-6 induces an androgen-like response in the LNCaP model system in our hands, the ability of increasing doses of IL-6 to modulate steady-state mRNA expression of the androgen-responsive PSA (28) in LNCaP cells was determined. In the absence of androgens, IL-6 caused a dose-dependent increase of PSA mRNA levels (Fig. 1). The mean PSA mRNA levels increased ~2- and 3.5-fold at 10 and 25 ng/ml of IL-6, respectively. Thus, IL-6 increases steady-state mRNA expression of an androgen-responsive gene, which is consistent with the possibility that it may activate the AR.

To determine whether IL-6 increased steady state mRNA levels of androgen-responsive genes through increasing their transcription, the ability of IL-6 to activate that PSA promoter was determined. IL-6 induced more than a 4-fold increase of PSA promoter activity (Fig. 2A). To next evaluate whether IL-6-mediated transactivation extended to other androgen-responsive promoters, we measured the ability of IL-6 to activate the MMTV(22) and the mouse SLP (24) promoters. IL-6 (10–25 ng/ml) transactivated both the MMTV and SLP promoters approximately 6-fold and 3- to 4-fold, respectively (Fig. 2B and 2C) demonstrating that IL-6 can induce a variety of androgen-responsive genes. To determine whether IL-6-mediated

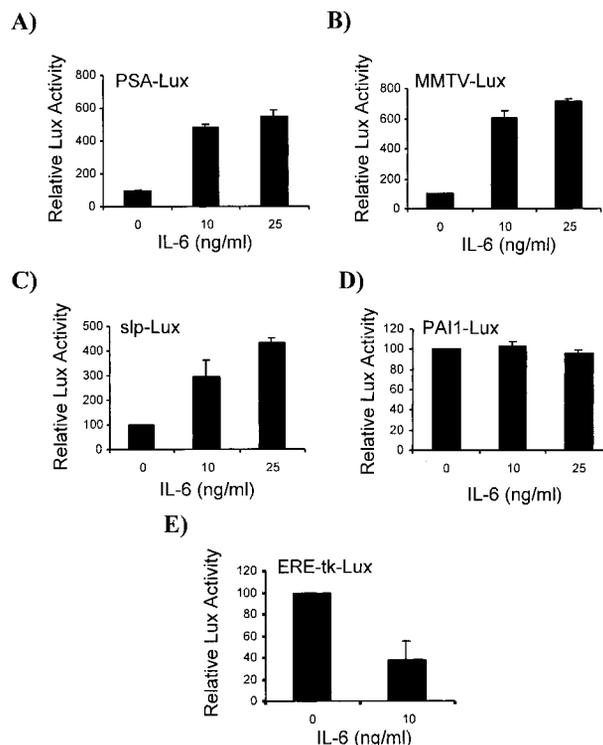


Fig. 2 IL-6 activates androgen-responsive promoters in the absence of androgen in LNCaP cells *in vitro*. LNCaP cells were cotransfected with pRL-CMV (for normalization using the dual lux assay) and one of the following: (A) p1.5kPSA-Lux, containing the proximal -1.5-k bp of the PSA promoter; (B) pMMTV-Lux, containing 150 bp of the MMTV promoter; (C) pc' Δ 9(slp)-Lux, containing -900 bp of the SLP promoter; (D) pPAI-1-Lux, containing a human type-1 plasminogen-activator-inhibitor gene promoter (-800/+22); or (E) pERE-tk-Lux, containing tandem repeat of estrogen response elements upstream of the thymidine kinase promoter. Cells were then incubated in charcoal-stripped medium with the addition of 0, 10, or 25 ng/ml IL-6 for 24 h. After incubation, lux levels were measured using a luminometer. Data are reported as the mean (\pm SE) lux activity relative to the basal activity, which was set as 100%, from three independent experiments.

transactivation was a nonspecific phenomenon, we evaluated the ability of IL-6 to induce several androgen nonresponsive genes; the human type-1 plasminogen activator inhibitor gene promoter and the thymidine kinase (tk) promoter downstream of a tandem ER response elements. In contrast to the androgen-responsive genes, these promoters were not activated by IL-6 (Fig. 2D and 2E). Taken together, these data suggest that IL-6 transactivates androgen-responsive genes with some degree of specificity.

The observation that IL-6 activated several different androgen-responsive genes suggests that there is a common mechanism through which IL-6 mediates this effect. A candidate target for IL-6-mediated transactivation of androgen-responsive promoters is the AR. Thus, to test if IL-6 transactivates androgen-responsive promoters through the AR, we incubated the cells with BIC (Casodex), an antiandrogen that is able to completely abolish AR function (29). BIC completely blocked the IL-6-mediated induction of the PSA and MMTV promoters (Fig. 3A) suggesting that IL-6 induces these genes through the AR. To further support IL-6's requirement for AR, the ability of

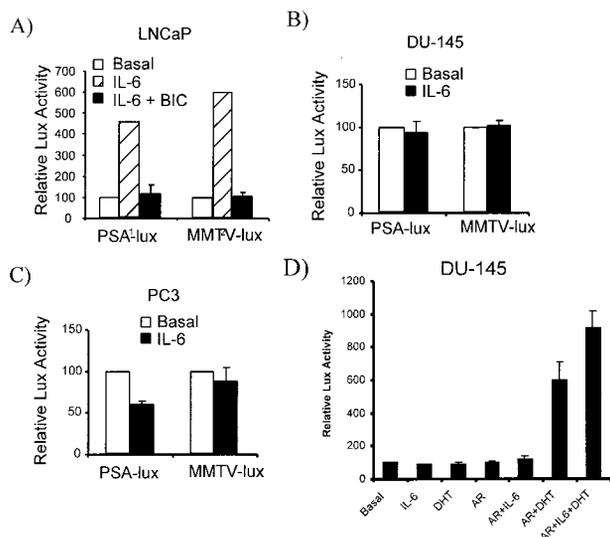


Fig. 3 IL-6-induced transcriptional activity requires an active AR. (A) LNCaP cells, (B) DU-145 cells, and (C) PC3 cells were cotransfected with pRL-CMV (for normalization using the dual lux assay) and either p1.5kPSA-Lux or pMMTV-Lux. The cells were then untreated (*Basal*) or treated with either IL-6 (10 ng/ml) or IL-6 (10 ng/ml) and 20 μ M BIC for 24 h as indicated. After incubation, lux levels were measured using a luminometer. *D*, DU-145 cells were cotransfected with pRL-CMV and p1.5kPSA-Lux. *Lanes 4–7*, cells were additionally cotransfected with 50 ng of pCMV-AR (*AR*). Cells were then incubated in charcoal-stripped media with the addition of 10 ng/ml IL-6, or 10 nM DHT, or both (as indicated) for 24 h. Data are reported as the mean (\pm SE) lux activity relative to the basal activity, which was set as 100%, from three independent experiments.

IL-6 to activate the PSA and MMTV promoters in two AR-negative CaP cell lines that express the IL-6 receptor α and β (gp 130) chains (4, 5, 21), DU-145 and PC3, was determined. IL-6 did not induce PSA or MMTV promoter activity in these cell lines (Fig. 3B and 3C). When AR was transiently expressed in DU-145 cells, IL-6 alone still did not induce the PSA promoter (Fig. 3D, *Lane 5*) although DHT alone did (Fig. 3D, *Lane 6*). However, IL-6 increased the androgen-induced PSA promoter activity (Fig. 3D, *Lane 7*). Similar results were obtained for activation of the MMTV promoter (data not shown). Collectively, these data suggest that AR is required, but not sufficient, for the IL-6-mediated activation of androgen-responsive promoters.

Several nonsteroidal substances induce AR activity through a variety of signal transduction pathways (18, 30–34). IL-6 activates PI-3 kinase pathway in several cell types including LNCaP cells (25, 26, 35, 36). Furthermore, IL-6 requires the growth factor receptors ErbB2 and -3 for signaling in LNCaP cells (20). To determine whether IL-6-induced androgen-responsive gene activation is mediated through these signal transduction pathways, the ability of kinase inhibitors to abrogate IL-6-mediated activation of the PSA promoter was evaluated. SB202190 (a p38 MAPK inhibitor), PD98059 (a MEK1/2 inhibitor), and tyrphostin AG879 (a ErbB2 MAPK inhibitor) but not wortmannin (a PI-3-kinase inhibitor), blocked IL-6-mediated induction of the PSA promoter activity (Fig. 4A). LY294002 (a PI-3-kinase inhibitor) has been shown to cause

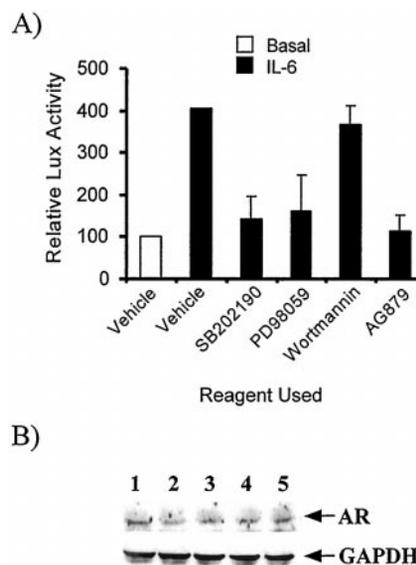


Fig. 4 PSA promoter induction by IL-6 was blocked by specific inhibitors to signal transducers. *A*, LNCaP cells were cotransfected with pRL-CMV (for normalization using the dual lux assay) and p1.5kPSA-Lux. The cells were then incubated with vehicle, SB202190 (5 μ M), PD98059 (15 μ M), wortmannin (100 nM), or tyrphostin AG879 (10 μ M) for 30 min prior to IL-6 administration (10 ng/ml) for 24 h. After incubation, lux levels were measured using a luminometer. Data are reported as the mean (\pm SE) lux activity relative to the basal activity, which was set as 100%, from three independent experiments. *B*, the immunoblot of AR and GAPDH from 40 μ g of cell lysate. LNCaP cells were cultured in medium containing IL-6 (*Lane 1*) or IL-6 plus pretreatment of the inhibitor reagents described above (*Lanes 2–5*, respectively).

program cell death to LNCaP (37). To ensure that the negative result that we obtained in Fig. 4A was not derived from cell death, we examined the cell viability by trypan blue exclusion on cells that followed the same treatment. We found no significant variation of the living cell number among different treatment group (data not shown). Because, in the report of Carsen *et al.*, addition of growth factor or serum in addition to LY294002 protected the LNCaP cells from apoptosis, it is plausible that the addition of IL-6 after cells were given the inhibitors may save cells from the cytotoxic effect. Also, to ensure that this response was not attributable to kinase inhibitor-mediated alteration of AR levels, we measured AR protein in the cells. The level of AR from cells treated with IL-6 and kinase inhibitors was the same as to that of IL-6 alone (Fig. 4B), except for a slight reduction of AR level from cells treated with SB202190 (Fig. 4B, *Lane 2*). Because MAPK inhibition blocked IL-6-mediated activation of the PSA promoter, we next assessed whether activation of MAPK could induce PSA promoter expression. Accordingly, we treated cells with EGF (a MAPK activator known to activate ER-dependent promoter activities; Ref. 38). EGF (100 ng/ml) did not induce PSA promoter activity in either the absence or the presence of transgenic AR in DU145 cells (data not shown). However, EGF (100 ng/ml) induced ERE-tk-Lux activity by 30-fold in the presence of transgenic ER α , which demonstrated that EGF was functional (data not shown). Together, these data suggest that MAPK activation is

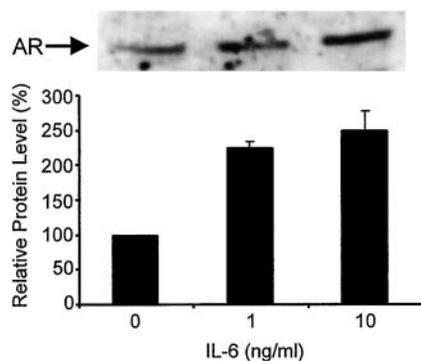


Fig. 5 Total AR levels from LNCaP cells are up-regulated by IL-6. LNCaP cells were treated with 0, 1, or 10 ng/ml of IL-6 for 24 h in charcoal-stripped media. Total cellular protein was then collected and subjected to Western blot analysis (200 μ g/lane) using an antihuman AR NH₂-terminal antibody. Bands were measured by densitometry. The blot shown here is the result of a typical experiment. Data are reported as mean (\pm SE) percentage of AR protein level relative to untreated cells, which was set as 100%, from three independent experiments.

necessary, but not sufficient, for IL-6-mediated activation of the PSA promoter.

Hobisch *et al.* reported that IL-6 induces LNCaP cell androgen responsiveness in the presence of low doses of androgen (18). One mechanism, through which IL-6 may increase androgen responsiveness in an AR-dependent fashion, as the current data demonstrate, is through induction of AR expression. Thus, the ability of IL-6 to increase AR expression in LNCaP cells was determined. The average AR protein amount doubled in the presence of 1 ng/ml of IL-6, whereas a 2.5-fold increase of mean AR level was observed when the IL-6 concentration was increased an order of magnitude (Fig. 5, *Lanes 2 and 3*). These data are consistent with the possibility that IL-6 sensitizes LNCaP cells to androgen through increased AR expression. However, the AR typically translocates to the nucleus to exert its function on gene expression (39). Accordingly, we determined whether IL-6 induces nuclear AR translocation. In the absence of DHT or IL-6, AR was detected primarily in the cytoplasm (Fig. 6, *A and D*). IL-6 induced a nuclear AR pattern similar to that of DHT-induced nuclear AR expression (compare Fig. 6, *B, C, E, and F*). In agreement with the immunofluorescent staining and the GFP tagging results, IL-6 increased AR levels in nuclear extract from LNCaP cells (Fig. 6*G*). Taken together, these data suggest that IL-6 induces androgen-responsiveness through increasing total AR protein levels that results in increased nuclear AR.

It is plausible that IL-6 induces AR expression through transcriptional or translational mechanisms. To determine whether IL-6 increases AR expression levels through transcriptional mechanisms, the ability of IL-6 to modulate AR mRNA levels was initially determined. IL-6 at 10 and 25 ng/ml increased steady-state AR mRNA levels 3-fold and 4-fold, respectively (Fig. 7). To evaluate whether IL-6 increases steady-state AR mRNA levels through transcription, the ability of IL-6 to activate the AR promoter was determined. IL-6 activated the AR promoter in a dose-dependent fashion (Fig. 8). Specifically, IL-6 at 5 and 25 ng/ml induced 3- and 3.5-fold activation, respec-

tively, of the AR promoter (Fig. 8, *Lanes 2 and 3* compared with *Lane 1*). These data correlated well with the magnitude of increased steady-state AR mRNA levels induced by IL-6. To determine whether the IL-6-induced increase of AR levels is sufficient to increase androgen activity in the absence of ligand, we overexpressed AR in LNCaP cells (Fig. 9, *Lanes 2 and 3*). Surprisingly, overexpression of AR diminished basal PSA mRNA levels (Fig. 9). These results demonstrate that increased AR levels alone are not sufficient to mediate IL-6 induction of androgen response in the absence of androgen, which suggests that IL-6 activates the AR in addition to increasing AR expression.

DISCUSSION

Induction of AR gene transcription is a relatively understudied area. Prior to the current study, only androgens were reported to activate the AR gene promoter. Thus, the present report is the first description of a non-androgen inducer of AR gene transcription, namely IL-6. In addition to increasing AR gene expression, this study demonstrated that IL-6 activates the AR in the absence of androgen. The ability of IL-6 to activate AR requires MAPK activity; however, MAPK alone is not sufficient to activate the AR. Taken together, these results suggest that IL-6 may promote androgen-independent prostate cancer progression through both increasing AR levels and increasing AR activity.

Elevated serum IL-6 expression is associated with the morbidity and progression of prostate cancer (40). Furthermore, IL-6 induces prostate cancer cell proliferation and protects prostate cancer cell lines from chemotherapeutics *in vitro* (41, 42). Our finding that IL-6 induced three different androgen-responsive promoters (PSA promoter, MMTV promoter, and SLP promoter) is consistent with a previous report that IL-6 activated an ARE-driven minimal promoter reporter vector (18). Our study extended this previous report by evaluating the affect of IL-6 on the ARE in the context of several natural androgen-responsive promoters, thus approximating the natural promoter activity better than isolated AREs. Our observations suggest that one mechanism through which IL-6 may contribute to prostate cancer progression is the ability to activate a general androgen response in prostate tumors. Such a response could be associated with increased tumor proliferation in the absence of androgens, which would contribute to the development of androgen independence.

The observation that IL-6 activated several androgen-responsive promoters but not androgen-nonresponsive promoters suggests that IL-6 has a degree of specificity for inducing androgen-like response. One potential mechanism that could account for such a response is the induction of AR activity. The observations that BIC blocked the effect of IL-6 and that IL-6 could not activate the PSA and MMTV promoters in AR-negative CaP cells support this hypothesis. Two non-mutually exclusive mechanisms that could account for the ability of IL-6 to increase AR action are increased AR levels and increased AR function.

The observation that IL-6 induced androgen-responsive genes in the absence of androgens through an AR-dependent mechanism suggested that IL-6 activates AR function. This

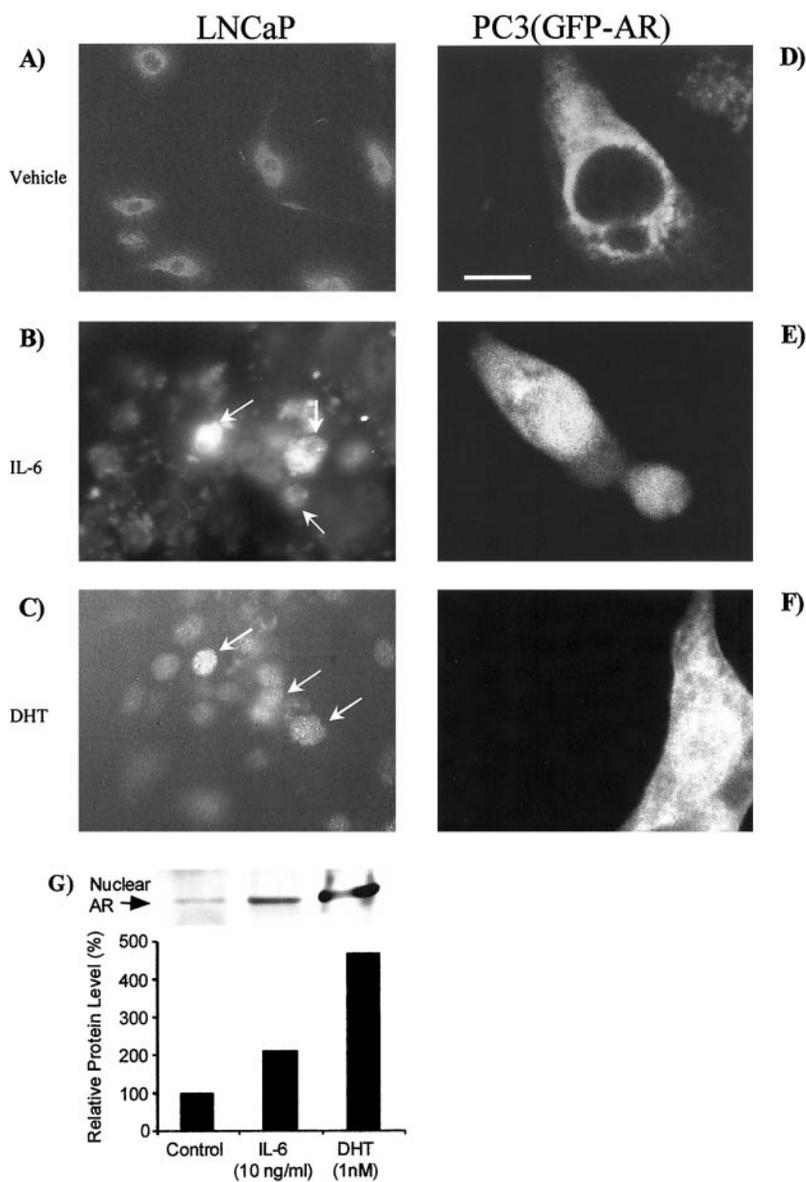


Fig. 6 IL-6 increases nuclear AR levels in prostate cancer cells. LNCaP cells and GFP-AR-expressing PC3 cells were incubated with: **A** and **D**, vehicle alone; **B** and **E**, IL-6 (10 ng/ml); or **C** and **F**, DHT (1 nM). After 24 h, they were subjected to immunofluorescent staining with an anti-AR antibody or confocal microscopy. *Arrows*, nuclear staining. **A**, $\times 400$; **B**, $\times 630$; **C**, $\times 400$. *Bar*, 15 μm . **G**, LNCaP cells were treated with medium alone, IL-6 (10 ng/ml), or DHT (1 nM) for 24 h; then nuclear proteins were collected and subjected to Western blot analysis (20 $\mu\text{g}/\text{lane}$) and probed with anti-AR antibody. Bands were measured by densitometry. The blot shown here is the result of a typical experiment. Data are reported as percentage of AR protein relative to untreated cells, which was set at 100%.

observation is consistent with several other nonandrogenic compounds that have been reported to stimulate AR. For example, several substances that bind to membrane receptors up-regulate the activity of AR (30–34). Furthermore, that IL-6 increased nuclear AR in the absence of androgen suggests that IL-6 may mediate its effect in part through unmasking the nuclear localization sequence. However, our observation that IL-6 did not induce androgen-responsive genes in the DU145 cells transfected with the AR is in apparent conflict with these findings. Taken together, these observations suggest that the AR is required but not sufficient for IL-6 to mediate androgen-like activity. It is possible that DU145 cells are deficient in some cofactor required for IL-6 to mediate its androgen-like activity.

Several signal transduction molecules are potential mediators of the ability of IL-6 to activate AR. For example, the observations that several MAPK inhibitors blocked IL-6-mediated

androgen-responsive promoter activation suggest that IL-6 induces AR activity through a MAPK-dependent pathway. These data are consistent with the ability of IL-6 to induce the Ras signal transduction pathways (43), which depends on Raf, MEK, and MAPK. These data are also consistent with the previous report that IL-6 requires the growth factor receptors ErbB2 and -3 to mediate signaling in LNCaP cells (20). However, the observation that the PI-3-kinase inhibitor, wortmannin, had no effect on IL-6-mediated PSA promoter induction suggests that this pathway is not important for androgen responsiveness, although IL-6 activates the PI-3-kinase pathway in several cell types including LNCaP cells (25, 26, 35, 36). Finally, the recent report that the inhibition of IL-6-mediated activation of STAT3 diminishes the ability of IL-6 to induce androgen-like activity suggests that the Jak/STAT pathway is an important component of IL-6-mediated activation of the AR

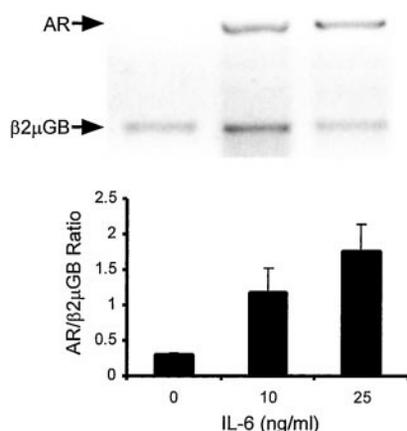


Fig. 7 IL-6 induces AR mRNA levels in LNCaP cells. LNCaP cells were treated with IL-6 (0, 10, and 25 ng/ml) for 24 h. Total RNA was collected and subjected to semiquantitative RT-PCR using primers for AR (AR) and β 2-microglobulin (β 2 μ GB). PCR products were measured by densitometry. Data are reported as the mean (\pm SE) ratio of AR: β 2 μ GB mRNA from three independent experiments. The gel shown is the result of a typical experiment.

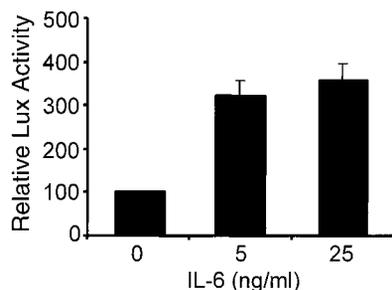


Fig. 8 IL-6 stimulates AR gene promoter activity in LNCaP cells *in vitro*. LNCaP cells were cotransfected with pRL-CMV (for normalization using the dual lux assay) and the reporter plasmid, p2.3kAR-Lux. Cells were then treated with IL-6 (0, 5, 25 ng/ml) for 24 h. After incubation, lux levels were measured using a luminometer. Data are reported as the mean (\pm SE) lux activity relative to the untreated cells, which was set as 100%, from three independent experiments.

(19). These previous reports, taken together with our current data, suggest that both MAPK and Jak/STAT signal transduction pathways are required to mediate the effect of IL-6 on AR function.

In addition to increased AR function, the finding that IL-6 increased AR expression at both mRNA and protein levels suggests that IL-6 enhances androgen activity by up-regulating the AR levels. This is consistent with the heterogeneous expression of AR that occurs as prostate cancer progresses (44–46). Furthermore, it has pathophysiological implications because there are low levels of androgens even after androgen ablation therapy (47). Specifically, if IL-6 increases AR levels in prostate cancer cells, then it follows that these cells may have increased sensitivity to the low levels of androgens in the androgen-ablated patient, enabling these cells to respond to androgens. In effect, what appears to be an androgen-independent tumor, based on its progression in an androgen-ablated patient, would

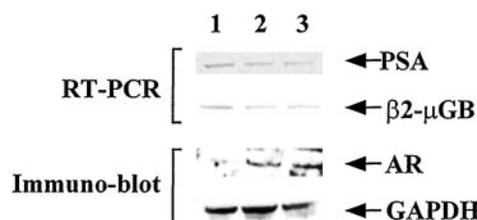


Fig. 9 Increased AR levels in LNCaP cells cause reducing PSA expression. LNCaP cells were transfected with empty vector (Lane 1), 0.4 μ g or 2 μ g of AR plasmid (Lanes 2 and 3). Total RNA was collected and subjected to semiquantitative RT-PCR using primers for PSA and β 2-microglobulin (β 2 μ GB). PCR products were resolved by electrophoresis. Protein from the same cell lysate 40 μ g were resolved by SDS-PAGE gel electrophoresis followed by AR and GAPDH immunoblotting.

really be hypersensitive to androgens. This hypothesis is consistent with the fact that the LNCaP cells, which are androgen responsive, were originally isolated from a patient in whom androgen ablation failed (16, 17).

The clinical observations that elevated IL-6 levels are frequently associated with androgen-independent prostate cancer have predicted an important role for IL-6 signaling in prostate cancer androgen-independent progression (40, 48–51). For example, serum IL-6 levels were shown to predict survival in prostate cancer patients (49). IL-6 levels in the serum of these patients range between 10 and 700 pg/ml, with occasional findings of >1 ng/ml (40, 48–51). In our study, we used nanogram levels of IL-6, which may reflect the IL-6 levels found in the tumor microenvironment. This postulation is supported by the observations that several prostate cancer cell lines produce nanogram levels of IL-6⁴ (5, 52). However, because of the wide range of IL-6 levels in men afflicted with prostate cancer, the *in vitro* application of IL-6 at the nanogram level requires cautious interpretation in terms of its bearing on *in vivo* pathophysiology. Moreover, from our data, which showed that IL-6 very likely contributes to the growth of advanced-prostate-cancer growth and that BIC abolishes the effect of IL-6, one might expect that this antiandrogen will be a powerful therapeutic agent in treating the advanced-prostate-cancer patient. Unfortunately, trials of BIC monotherapy have not demonstrated efficacy in the clinical setting (reviewed in Ref. 53). Because of the fact that the cancer tissue is heterogeneous, it is likely that there is a subpopulation of cancer cells that can survive via alternative mechanisms to which IL-6 may contribute independent of the AR signaling pathway.

The demonstration of IL-6-induced activation of the AR promoter and the subsequent increase of AR mRNA and protein is the first report of non-androgen-mediated induction of the human AR promoter. Other than IL-6, only androgens have been reported to activate the human AR promoter (54–56). Grad *et al.* (57) demonstrated the regulatory motif within the AR gene-coding region required for AR auto-up-regulation. Whether or not IL-6 uses this same motif is currently unknown.

⁴ Unpublished observations.

Furthermore, the *trans*-acting factors and *cis*-acting promoter sites through which IL-6 mediates induction of the AR promoter are currently unknown. The induction of AR mRNA and protein expression described thus far has been rare (58–60). Thus, our data provide a new mechanism through which AR levels are controlled.

Advanced CaP is associated with increased serum IL-6 levels (50). The source of IL-6 in the CaP patients is not known. However, we have previously demonstrated that androgen down-regulates IL-6 expression in LNCaP cells (61). Furthermore, we and others have shown that orchiectomy induces IL-6 expression in mice (62, 63). Thus, it follows that androgen deprivation may account for the increased serum IL-6 levels observed in patients with advanced prostate cancer. These observations, taken together with results from the present study, suggest that androgen deprivation may induce IL-6 expression in prostate cells, which, in turn, will induce androgen-like activity in the CaP cells. This could lead to androgen-independent tumor growth. Intriguingly, it is possible that, as IL-6 induces androgen-like activity, this will in turn create a negative feedback on IL-6 production.

In summary, our study provides evidence that IL-6 increases androgen-like action by up-regulating the AR expression in CaP cells. Furthermore, our data suggest that IL-6 activates the AR in the absence of androgen. Together, these mechanisms can account for the contribution of IL-6 to the progression of prostate cancer. Furthermore, these results suggest that androgen-deprivation therapy may promote the progression of CaP to an androgen-independent state through increasing expression of IL-6.

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