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Interleukin-6 differentially regulates androgen receptor transactivation via PI3K-Akt, STAT3, and MAPK, three distinct signal pathways in prostate cancer cells☆

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Abstract

The effects of IL-6 on prostate cancer cells are well documented yet remain controversial. Some reports suggested that IL-6 could promote prostate cancer cell growth, while others showed that IL-6 could repress prostate cancer cell growth. Here, we systemically examined various IL-6 signaling pathways in prostate cancer cells and found that IL-6 could go through at least three distinct pathways to modulate the functions of androgen receptor (AR), a key transcriptional factor to control the prostate cancer growth. Our results show that IL-6 can enhance AR transactivation via either the STAT3 or MAPK pathways. In contrast, IL-6 can suppress AR transactivation via the PI3K-Akt pathway. Co-existence of these various signaling pathways may result in either additive or conflicting effects on AR transactivation. Together, our results indicate that the balance of these various pathways may then determine the overall effect of IL-6 on AR transactivation.
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Keywords: AR; Transactivation; IL-6; PI3K; MAPK; STAT3

Prostate cancer is the second most prevalent cancer in males in the United States. There is evidence that IL-6 may play an important role in metastatic prostate cancer. It has been demonstrated that all of the commonly used prostate cancer cell lines (PC-3, DU145, and LNCaP) express receptors with a high affinity for IL-6 [1–4]. In addition, prostate cancer cell lines, PC-3 and DU145, have been demonstrated to secrete varying amounts of IL-6, whereas the hormone dependent cell line, LNCaP, does not secrete IL-6 [2–4]. Clinical data show that serum IL-6 levels are elevated in men with hormone-refractory prostate cancer and that these high serum IL-6 levels are accompanied by high levels of serum prostate specific antigen (PSA) [5]. However, proliferation studies of IL-6 in LNCaP cells have resulted in contrasting results. Addition of exogenous IL-6 to the culture media of LNCaP cells showed that cell growth was inhibited in a dose-dependent manner [2,6,7]. In contrast, other reports revealed cell growth stimulation after treatment with IL-6 [3,8,9]. The reasons for these differences have not been clarified to date, but suggest that IL-6 may exert divergent effects in human prostate cancer.

The androgen receptor (AR) is a 110-kDa nuclear protein that consists of several domains, including transactivation, DNA binding, nuclear localization, dimerization, and ligand binding domains [10–13]. The AR is expressed in normal prostate tissue and prostate cancers, and is a key transcription factor to control prostate cell growth. Activation of the AR in prostate cancer is being intensively investigated. After a ligand

☆ *Abbreviations:* AR, androgen receptor; PI3K, phosphatidylinositol 3(OH)-kinase; IL6, interleukin 6; wtAR, wild-type AR; DHT, 5α-dihydrotestosterone; MMTV, mouse mammary tumor virus; PSA, prostate specific antigen; luc, luciferase.

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binds to AR, the ligand–receptor complex translocates into the nucleus and binds to specific androgen response elements (AREs) [14,15]. Some evidence shows that the AR could also be stimulated in the absence of its cognate ligand by a number of nonsteroidal compounds, such as various growth factors and protein kinase pathways [16,17].

Recent studies revealed that IL-6 could activate AR transactivation in an androgen-independent manner in LNCaP cells [18–20]. The PI3K pathway has been reported to be a major contributor in the signaling of IL-6, however, the role of PI3K in the activation of AR by IL-6 is controversial. Some studies suggested that PI3K may play a role in the activation of AR by IL-6, but another report showed that IL-6-mediated AR activation was not dependent on the PI3K pathway [18,21–24]. Moreover, although IL-6 is able to activate AR transactivation in an androgen-independent manner in LNCaP cells, IL-6 alone did not induce PSA expression and mouse mammary tumor virus (MMTV) promoter activity in PC-3 and DU145, in which AR was transiently expressed [18]. These results suggest that IL-6 may have distinct pathways in various prostate cancer cells. Accordingly, in the current report, we explored the availability of various IL-6 signaling pathways in various prostate cancer cells and compared what pathway is dominant in these various cell lines.

Materials and methods

Materials. pCDNA3-cAkt (a constitutively active Akt with a deletion at amino acids 4–129 replaced with a consensus myristylation domain) and pCDNA3-dAkt (a kinase deficient mutant, K179A) were from Dr. Robert Freeman [25]. pSG513-STAT3 and pSG513-STAT3β (a dominant-negative STAT3 with a point mutation) were from Dr. Rolf P. de Groot [26]. LY294002, U0126, and PD98059 were from Calbiochem and DHT was from Sigma. pCMV-AR, pSG5-AR, MMTV-luciferase (MMTV-luc) promoter, and a reporter containing 4 copies of ARE promoter-luciferase ((ARE)4-luc), pRL-SV40, and pRL-TK have been previously described [11]. Phospho-p44/42 MAP kinase (Thr202/Thr204) antibody and p44/42 MAP kinase antibody were purchased from Cell Signaling Technology.

Cell culture and transfections. The human prostate cancer PC-3 and DU145 cells were maintained in Dulbecco's minimum essential medium containing penicillin (25 U/ml), streptomycin (25 μg/ml), and 10% fetal calf serum (FCS). The human prostate cancer LNCaP cells were maintained in RPMI-1640 with 10% FCS.

Luciferase reporter assays. The cells were plated at 8×10^4 on 24-well plates and incubated with RPMI 1640 or Dulbecco's modified Eagle's medium containing 10% FBS for 24 h. Transfection was performed by SuperFect (Qiagen) according to the standard protocol. In brief, the total amount of plasmid DNA was adjusted to 1 μg/well by addition of control plasmid. After 3 h transfection, the medium was replaced with serum-free medium or 10% charcoal–dextran treated FBS and treated with DHT, IL-6, or inhibitors. The cells were washed with PBS and harvested after 24 h. Cell lysates were prepared and used for luciferase assay according to the manufacturer's instructions (Promega). The results were obtained from at least three sets of transfection and presented as means ± SD.

PI3K activity assay. PI3K activity was determined as previously described [27]. Briefly, cells were washed twice with ice-cold PBS and lysed in RIPA buffer. The lysates were centrifuged and the protein content was determined. Five-hundred microgram aliquots from each sample were immunoprecipitated with 40 μl aliquots of pre-conjugated, monoclonal anti-phosphotyrosine (PY20) agarose beads (Santa Cruz, CA) by incubating overnight at 4 °C in 500 μl of immunoprecipitation buffer (190 mM NaCl, 50 mM Tris–HCl, pH 7.4, 6 mM EDTA, and 2.5% Triton X-100). All subsequent steps were exactly as described [27]. Briefly, 30 μl aliquots from the kinase reaction assays were separated on thin layer Silica Gel 60 chromatography plates (EM Separations Technology), dried at room temperature, and autoradiographed.

Western blot analysis. Protein samples were prepared by lysing cells over ice in ice-cold RIPA buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 0.1% SDS, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, 1 mM Na_3VO_4, and 1 mM NaF). Cell lysates were centrifuged at 14,000*g* at 4 °C for 15 min. Protein content was determined using the *DC*-protein assay kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (50 μg) from cell lysates were denatured in sample buffer, subjected to SDS–PAGE on 4–20% gels (Novex/Invitrogen, San Diego, CA), and transferred to nitrocellulose membranes. The blots were probed with specific primary antibodies as recommended by the suppliers. Appropriate HRP-conjugated secondary antibodies were used (1:5000) and visualized by enhanced chemiluminescence (Bio-Rad Laboratories, Hercules, CA).

Results

IL-6 differentially induces AR transactivation in various prostate cancer cells

We first investigated the effect of IL-6 on AR transcriptional activity in LNCaP cells by transient transfection with the MMTV-luc reporter plasmid. The region of the MMTV promoter that contains the AREs is required for androgen induction. As shown in Fig. 1A, IL-6 had minimal effect on MMTV-luc activity in the absence of DHT in the LNCaP cells. We then treated the LNCaP cells with a low concentration of DHT (0.1 nM) and a maximum induction (45-fold) of MMTV-luc reporter activity was obtained and addition of IL-6 (50 ng/ml) resulted in a 70-fold increase in MMTV-reporter activity relative to the control. However, in contrast to the LNCaP cells, co-transfection of MMTV reporter and wild-type AR with 1 nM concentration of DHT and 50 ng/ml of IL-6 in DU145 and PC-3 cells did not show the similar induction pattern as in LNCaP cells. In contrast, a slight suppressive effect on AR activity was observed (Figs. 1B and C), suggesting that the IL-6 signal may differentially modulate AR activity in various prostate cancer cells.

Synergistic increases in the induction of MMTV promoter activity by IL-6 with blocking of the PI3K pathway

The fact that AR target gene reporters were poorly induced in PC-3 and DU145 cells upon stimulation by

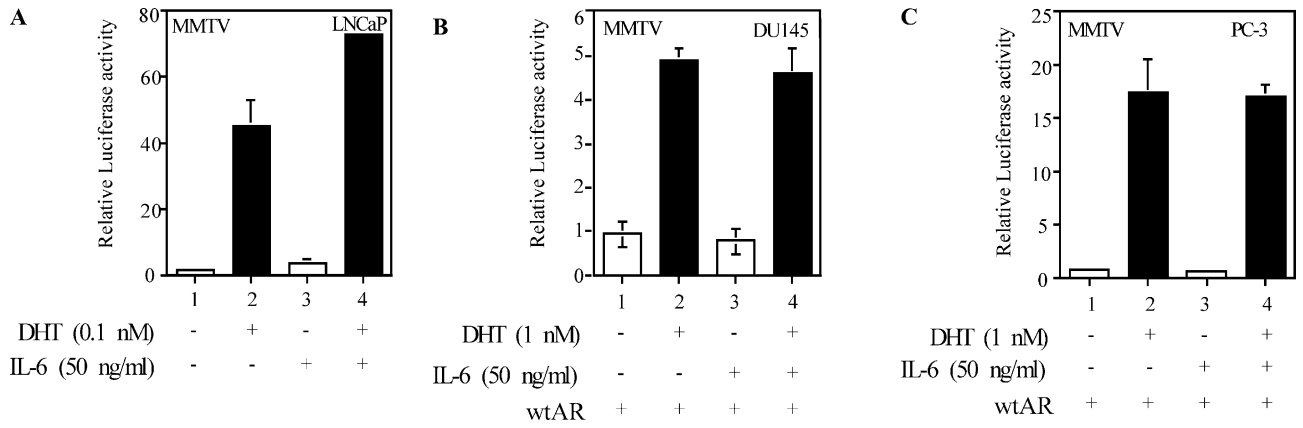

Fig. 1. IL-6 differentially modulated AR target gene in prostate cancer cell lines. (A) LNCaP cells were transiently transfected with MMTV-luc (300 ng/well) for 24 h and then incubated with 0.1 nM DHT or vehicle for additional 24 h under serum-free condition. (B,C) DU145 and PC-3 cells were co-transfected with wild-type AR and 300 ng/well MMTV-CAT or MMTV-luc, respectively, and then incubated with 1 nM DHT, 50 ng/ml IL-6, or vehicle for additional 24 h under serum-free condition, and then harvested. The internal control was 10 ng/well pRL-SV40 and the total amount of plasmid DNA transfected was adjusted to 1 μg/well by addition of the empty vector. The error bars represent the means ± SE of three independent experiments.

IL-6 led us to investigate the PI3K signaling in these two prostate cancer cell lines. We first investigated the effects of Δp85, the dominant-negative form of PI3K and P110, the active form of PI3K. As shown in Figs. 2A and B, addition of Δp85 enhanced AR transactivation in a dose-dependent manner. In contrast, addition of P110 repressed AR transactivation in a dose-dependent manner in DU145 cells. These data suggest that activation of PI3K pathway may result in the suppression of AR transactivation. We then investigated the effect of IL-6, the upstream regulator of PI3K, on the AR transactivation. As shown in Fig. 2C, addition of 1 nM DHT enhanced MMTV-chloramphenicol transferase (CAT) reporter activity 5-fold. Addition of IL-6, from 10 ng/ml to 100 ng/ml, slightly reversed this DHT-induced reporter activity (lanes 3, 4, and 5 vs lane 2). Addition of LY294002, a selective PI3K inhibitor, further enhanced DHT-induced reporter activity from 5-fold to 14-fold (lane 2 vs 6). Simultaneous addition of LY294002 and IL-6 synergistically enhanced reporter activity from 5-fold to 23–28-fold, suggesting that under blockade of PI3K condition, IL-6 may go through other pathways to stimulate AR activity, probably via MAPK signaling pathway, because MAPK inhibitor U0126 suppresses AR transactivation, which can further repress AR activity when the cells were treated with IL-6 (Fig. 2C, lanes 10–13). Together, results from Figs. 2A–C suggest that the lack of induction effect of IL-6 on AR transactivation may be due to enhancement of PI3K activity. To further confirm this hypothesis, we also assayed the PI3K activity upon addition of IL-6. As shown in Fig. 2D, addition of IL-6 enhanced PI3K activity in DU145 cells as well as PC-3 and LNCaP cells. In conclusion, data from Fig. 2 suggest that the IL-6 → PI3K signal pathway may play negative roles for the AR transactivation.

The effects of IL-6 → PI3K → Akt signal pathway on AR transactivation

As Akt is the downstream signal of IL-6 → PI3K,we were interested to see its effect on AR transactivation. As shown in Fig. 3A, addition of the constitutive-active form of Akt (cAkt) suppresses DHT-induced MMTV-luc reporter activity in PC-3 cells (lane 2 vs 3). In contrast, addition of dominant-negative form of Akt (dAkt) further enhanced DHT-induced MMTV-luc reporter activity (lane 2 vs 4). Similar results were also observed when we replaced PC-3 cells with DU145 cells (Fig. 3B). These data are consistent with Fig. 2 showing that IL-6 → PI3K → Akt signal pathway can suppress AR transactivation.

The effect of IL-6 → MAPK pathway on AR transactivation

MAPK represents another major downstream pathway to mediate IL-6 signal [19]. However, in contrast to the PI3K-Akt pathway that suppresses AR transactivation, our data show that addition of constitutive active MEK1 (cMEK1) further enhanced DHT-induced MMTV-luc reporter activity in PC-3 and DU145 cells (Fig. 3C, lane 2 vs 3; Fig. 3D, lane 2 vs 3). MAPK inhibitor U0126 exerted repression effect on DHT-induced AR transactivation (Fig. 3D, lane 4) in DU145 cells. Together, results from Figs. 3C and D suggest that MAPK may mediate IL-6 signal on AR transactivation in a positive manner.

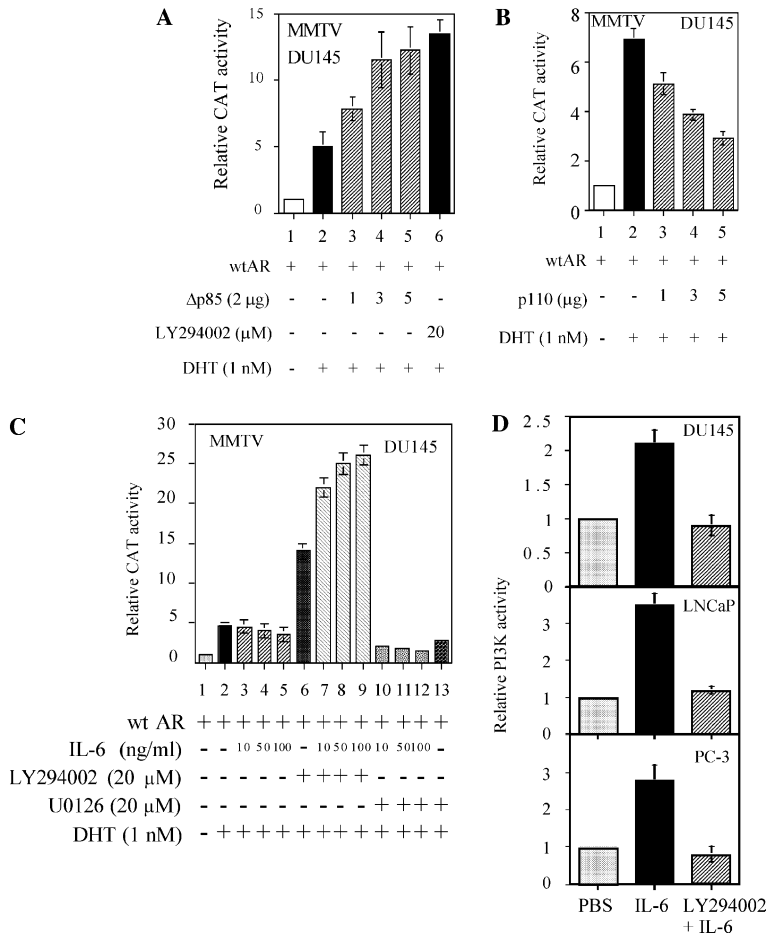

Fig. 2. Additive increases in the induction of MMTV promoter gene by IL-6 with blocking of the PI3K pathway. (A) Enhancement of AR transactivation through the inhibition of PI3K activity by Δp85 and LY294002. After 24 h transfection, the DU145 cells were treated with vehicle or LY294002 for 30 min prior to DHT treatment. The transactivation was measured by CAT activity. (B) Inhibition of AR transactivation by p110 in a dose-dependent manner. (C) Effect of IL-6 on AR transactivation. DU145 cells were transfected for 24 h with AR and MMTV-CAT reporter gene. After transfection, the cells were serum starved for 24 h, then 20 μM LY294002 or PD98059 was added to serum-free medium 30 min prior to IL-6 treatment. After 30 min of treatment with indicated concentrations of IL-6, DHT was added for another 24 h. The cells were then harvested and AR transactivation was measured by CAT activity. (D) Activation of PI3K activity by IL-6 in LNCaP, PC-3, and DU145 cells. After serum starvation for 24 h, LNCaP, PC-3, or DU145 cells were treated with 50 μg/ml IL-6 for 30 min, and then harvested. PI3K activity was measured as described in Experimental procedures.

The effects of combining PI3K-Akt and MAPK on AR transactivation

Fig. 3 suggests that PI3K-Akt and MAPK, which are distinct downstream signals of IL-6, can play opposite roles (suppression vs induction) on the AR transactivation. We were interested in determining their mutual influence on the AR transactivation. As shown in Fig. 4A, addition of cMEK1 alone further enhanced DHT-induced MMTV-luc reporter activity (lane 2 vs 4) and addition of cAkt then suppressed the cMEK-enhanced MMTV-CAT reporter activity (lane 4 vs lanes 6 and 8). Similar conclusions also occurred showing that cMEK1 reversed the cAkt-repressed AR transactivation in PC-3 cells (Fig. 4B). The interactions between two IL-6 downstream signals, MEK1 and cAkt, were further demonstrated using Western blot to assay the MAPK phosphorylation status. As shown in Fig. 4C, MAPK was phosphorylated upon stimulation of IL-6 (lane 2). This IL-6-induced MAPK phosphorylation was suppressed upon addition of U0126, the MEK1 inhibitor (lane 3 vs 4). Interestingly, if we replaced U0126 with LY294002, the inhibitor of cAkt, we found that the phosphorylation of MAPK increased significantly (lane 5 vs 6), suggesting that blocking of the IL-6 → PI3K → Akt pathway may be able to potentiate the IL-6 → MAPK pathway.

IL-6 potentiates STAT3 effect on enhancement of AR transactivation

The third major downstream signal of IL-6 is STAT3. As shown in Fig. 5A, addition of STAT3 enhanced

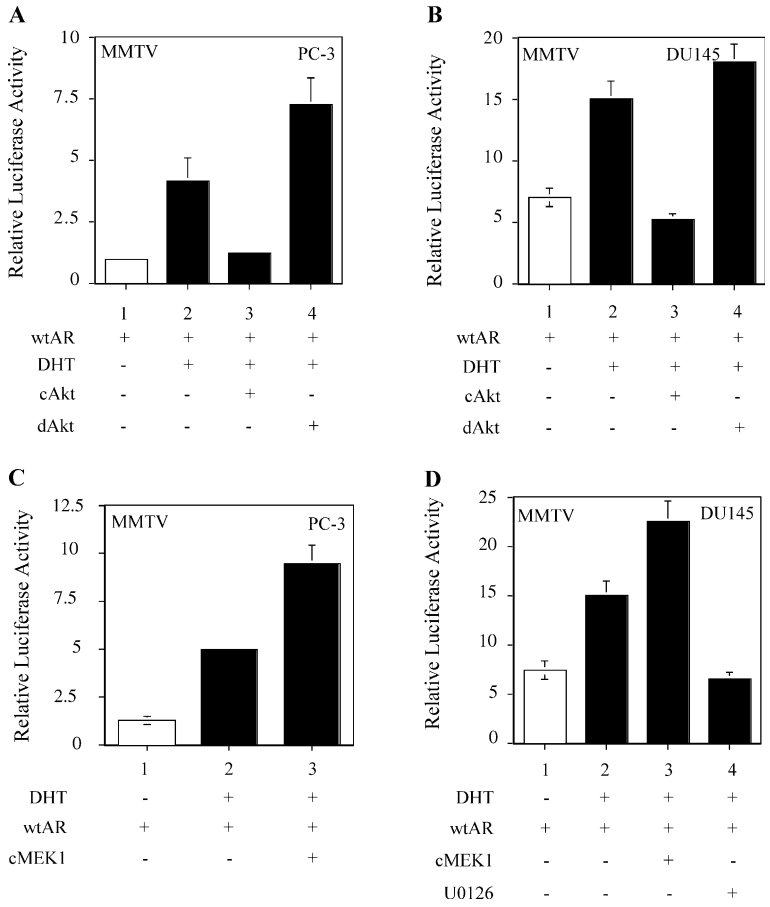

Fig. 3. cAkt suppresses, but cMEK1 enhances AR activity. PC-3 (A) and DU145 cells (B) were transfected with 50 ng pCMV-AR, 150 ng MMTV-luc, 2.5 ng pRL-SV40, 50 ng cAkt, or dAkt. Transfected cells were treated for 24 h with 10^{-9} M DHT or ethanol as vehicle controls. PC-3 (C) and DU145 cells (D) were transfected with 50 ng pCMV-AR, 150 ng MMTV-luc, 2.5 ng pRL-SV40, and 100 ng cMEK1. Transfected cells were treated for 24 h with 10^{-9} M DHT or ethanol as vehicle controls. In DU145 cells (D, lane 4), 20 μM U0126 was added with the DHT. Duplicate samples were analyzed for each data point.

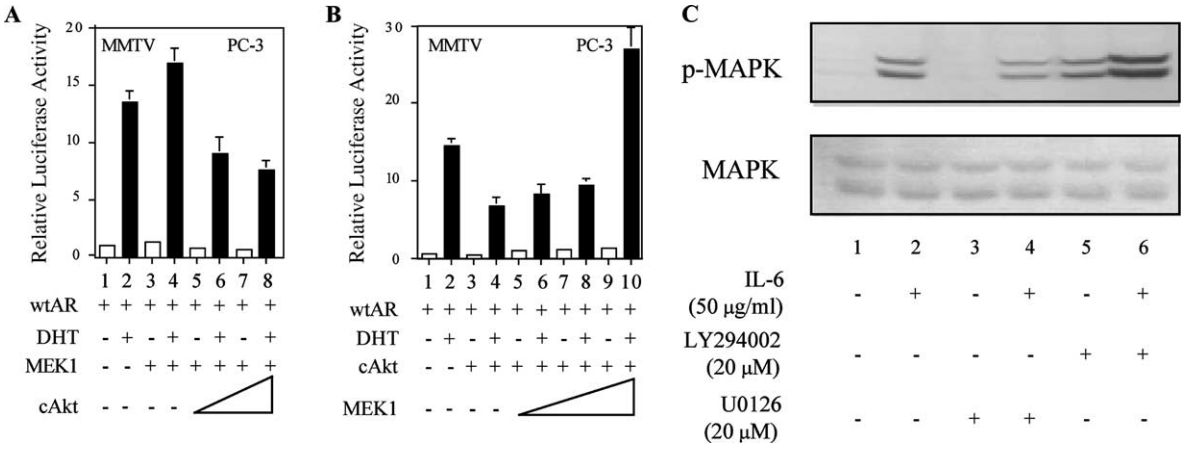

Fig. 4. cAkt and MAPK counteract with each other to modulate AR activity, and IL6 activates MAPK in DU145 cells. (A) PC-3 cells were transfected with 50 ng pSG5-AR, 150 ng MMTV-luc, 100 ng cMEK1, 5 ng pRL-TK, and doses of cAkt (50 and 150 ng). (B) PC-3 cells were transfected with 50 ng pSG5-AR, 150 ng MMTV-luc, 100 ng cAkt, 5 ng pRL-TK, and doses of cMEK1 (50, 150, and 300 ng). Cells were treated for 24 h with 10^{-9} M DHT or ethanol as vehicle controls. Duplicate samples were analyzed for each data point. (C) DU145 cells were treated with 50 ng/ml IL-6, 20 μM LY294002 or 20 μM U0126, or a combination as indicated for 30 min. The phosphorylation status of MAPK was determined by immunoblotting with phospho-p44/p42 MAPK monoclonal antibody (top). The loading control was carried out with anti-MAPK antibody (bottom).

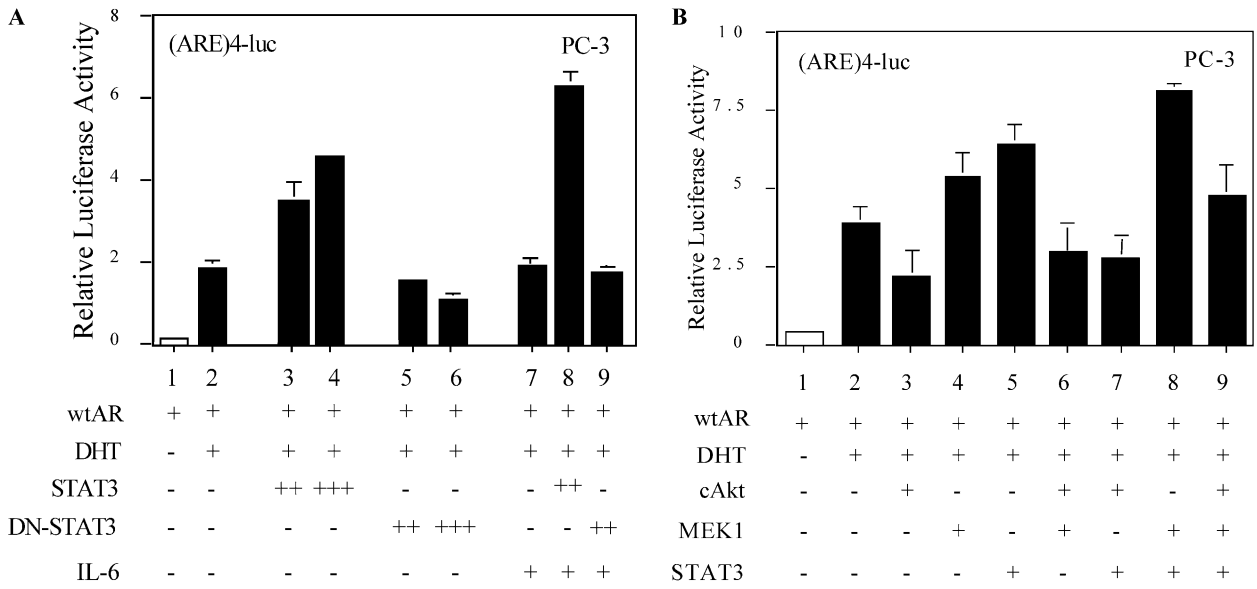

Fig. 5. IL-6 potentiated STAT3 effect on enhancement of AR transactivation and the effects of the co-existence of 3 IL-6 downstream signal pathways. (A) PC-3 cells were transfected with 50 ng pCMV-AR, 150 ng (ARE)4-luc, doses of STAT3 or DN-STAT3 (++, 50 ng and +++, 150 ng), and 5 ng pRL-TK. Transfected cells were treated for 24 h with 10^{-9} M DHT, ethanol, or 50 ng/ml IL6. Duplicate samples were analyzed for each data point. (B) PC-3 cells were transfected with 50 ng pCMV-AR, 150 ng (ARE)4-luc, 5 ng pRL-TK, and 50 ng of cAkt, cMEK1, and STAT3, or combinations of them. Transfected cells were treated for 24 h with 10^{-9} M DHT, ethanol, or 50 ng/ml IL6. Duplicate samples were analyzed for each data point.

DHT-induced AR transactivation in a dose-dependent manner and addition of dominant-negative STAT3 suppressed AR transactivation in PC-3 cells. Addition of IL-6 alone had little effect on the AR transactivation. Addition of IL-6 and STAT3, however, further potentiates STAT-induced AR transactivation (lane 4 vs 8). Together, results from Fig. 5A suggest that IL-6 can enhance AR transactivation through the STAT3 signaling pathway.

The effects of the co-existence of 3 IL-6 downstream signal pathways

To study the potential mutual influences of the 3 IL-6 downstream signal pathways (PI3K-Akt, MAPK, and STAT3) on AR transactivation, we cotransfected these 3 downstream mediators in different combinations in PC-3 cells. As shown in Fig. 5B, addition of cAkt alone suppressed DHT-induced AR transactivation (lane 3). Addition of MEK1 alone enhanced DHT-induced AR transactivation (lane 4). Addition of STAT3 alone enhanced DHT-induced AR transactivation (lane 5). Simultaneous addition of cAkt and MEK1 (lane 6) or cAkt and STAT3 (lane 7) results in the slight suppression of AR transactivation. In contrast, simultaneous addition of MEK1 and STAT3 results in further enhancement of AR transactivation (lane 8). Simultaneous addition of cAkt, MEK1, and STAT3 resulted in the slight enhancement of AR transactivation (lane 9). Together, results from Fig. 5B suggest that IL-6 effects on the AR transactivation may depend on the availability of its three downstream mediators.

Discussion

The role of cytokines in normal prostate biology and prostate cancer is still an emerging area of investigation. IL-6 is significantly elevated in many men with advanced hormone-independent prostate cancer and elevated IL-6 levels may constitute an independent prognostic marker for decreased survival [5]. Thus, it has been predicted that IL-6 signaling plays an important role in androgen-independent progression. IL-6 receptor is expressed in both prostate cancer tissues and prostate cancer cell lines, including the androgen-dependent prostate cell line LNCaP and androgen-independent PC-3 and DU145 cells [28,29]. Binding of IL-6 to its receptor results in activation of JAKs as well as their two major downstream signaling pathways, MAPK and STAT3, in LNCaP cells [8,18,19,29]. IL-6 can also activate the PI3K pathway in LNCaP and PC-3 cells [21–24]. Some reports observed that IL-6 is able to induce AR transactivation in an androgen-independent manner in LNCaP cells, but not in PC-3 and DU145 cells [18,19,30]. However, the mechanism of IL-6 induction of AR transactivation in LNCaP cells still remains largely unknown. To date, results revealed that the induction of AR target gene reporter activity by IL-6 was promoter-specific and cell type-specific. Some studies showed that

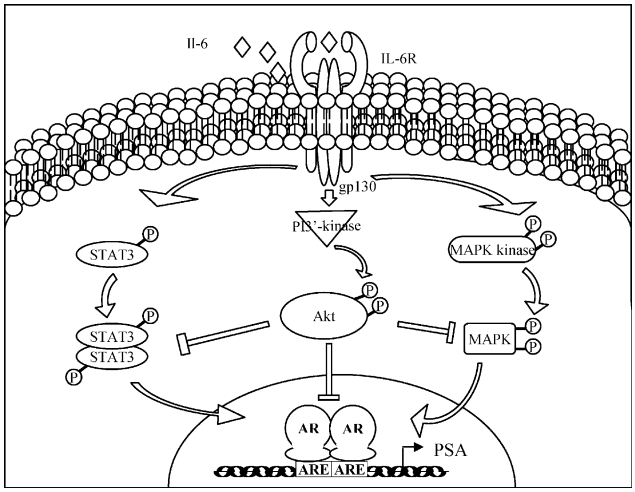

Fig. 6. A hypothetical working model of signal transduction pathways to AR activity upon stimulation by IL-6 in hormone-refractory prostate cancer cell lines. The IL-6 signaling cascade induces up-regulation of AR-regulated genes such as MMTV through the STAT3 and MAPK pathways, but down-regulation of MMTV through the PI3K-Akt pathway.

IL-6 could activate the AR gene promoter resulting in increased AR mRNA and protein level in LNCaP cells, suggesting that IL-6 enhances androgen activity by up-regulating the AR level [18]. However, other reports observed that the whole cell levels of AR were not increased by IL-6 [31].

The fact that AR target gene reporters were poorly induced in PC-3 and DU145 cells upon stimulation by IL-6 led us to investigate the discrepancies between LNCaP, PC-3, and DU145 cells. The observation that IL-6 was able to activate PI3K, STAT3, and MAPK pathways in PC-3 or DU145 cells, suggested that these three pathways may coordinate with each other to determine the effect of IL-6 on AR transactivation and prostate cancer growth. We found that IL-6 could enhance AR transactivation via the MAPK or STAT3 pathway. Alternatively, IL-6 could repress AR transactivation via the PI3K pathway. We also observed that the PI3K pathway could negatively influence the MAPK and STAT3 pathways, and the PI3K pathway may be more dominant compared to the MAPK and STAT3 pathways upon the stimulation by IL-6 in PC-3 or DU145 cells (Fig. 6). In LNCaP cells, although PI3K pathway is also an IL-6 signal mediator, it has been shown to not be a major signal transduction pathway for IL-6 effect on AR. However, we cannot rule out the possibility that in addition to the availability of these three signal transduction pathways, some mediators of IL-6 to modulate its effect on AR activity are deficient or different in PC-3 and DU145 cells. In conclusion, our data suggest that IL-6 may use multiple pathways to differentially regulate AR transactivation and/or AR-mediated cell growth in prostate cancer cells.

Acknowledgments

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