The Prostate 60:61 – 67 (2004)

Regulation of Interleukin-6-Mediated PI3K Activation and Neuroendocrine Differentiation by Androgen Signaling in Prostate Cancer LNCaP Cells

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BACKGROUND. Neuroendocrine (NE) differentiation in prostate cancer has been suggested to be one of the early events in the development of androgen independence. In the human prostate cancer LNCaP cell line, treatment with interleukin-6 (IL-6) induces NE-like differentiation, which is similar to the phenomena observed in advanced stages of prostate cancer progression. In this study, we investigate how androgen plays a role in IL-6-mediated NE differentiation in LNCaP cell line.

METHODS. Western blot, co-immunoprecipitation (co-IP), and GST pull-down assays were performed to detect the protein expression and protein–protein interaction. PI3K kinase assay was used to measure PI3K activity.

RESULTS. Addition of androgen blocks IL-6-mediated PI3K activation and NE differentiation in LNCaP cells. In vivo and in vitro protein interaction assays suggested that androgen receptor (AR) can directly interact with IL-6 transducer gp130. In addition, androgen treatment enhances the interaction between AR and gp130, interrupts the IL-6-induced gp130-mediated PI3K activation, which may lead to inhibition of IL-6-mediated NE differentiation in LNCaP cells.

CONCLUSIONS. Our results suggest androgen and AR can regulate IL-6-mediated LNCaP cell NE differentiation via directly modulating the IL-6-PI3K pathway. *Prostate 60: 61–67, 2004.* © 2004 Wiley-Liss, Inc.

KEY WORDS: **prostate cancer; differentiation; androgen; androgen receptor; IL-6; PI3K**

INTRODUCTION

Prostate cancer is the second leading fatal cancer in American men. Initially dependent on androgens for proliferation, prostate cancer eventually progresses to be androgen-independent. It has been reported that neuroendocrine differentiation (NE) in prostate cancer correlates with an unfavorable clinical outcome. Prostate tumors with a prominent NE component are typically androgen independent and highly aggressive. Many prostate cancers are infiltrated with NE-like cells. Evaluation of NE cell number has been correlated with androgen-independent disease and poor prognosis [1–3]. The molecular basis by which NE cells arise in prostatic tumors remains largely unknown. In vitro, IL-6 can induce NE differentiation in prostate cancer LNCaP cells [4–6].

IL-6 is a cytokine that not only regulates the immune response, but also modulates normal and cancer cell growth, differentiation, and survival. Elevated

Abbreviations: AR, androgen receptor; IL-6, interleukin-6; PI3K, phosphatidylinositol-3-kinase; NE, neuroendocrine; DHT, 5-dihydrotestosterone; HF, hydroxyflutamide; NSE, neuron-specific enolase.

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Grant sponsor: NIH; Grant number: DK-47258.

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Received 17 June 2003; Accepted 10 November 2003
DOI 10.1002/pros.20048
Published online 28 January 2004 in Wiley InterScience (www.interscience.wiley.com).

circulating levels of IL-6 have been associated with prostate tumor metastases and patient morbidity [1]. In prostate cancer LNCaP cells, IL-6 can regulate cell growth and NE differentiation, a phenotype including development of neuron-like morphological changes and increased expression of NE differentiation marker NSE (neuron-specific enolase) [7]. IL-6 exerts its activity on target cells by binding either to the membrane or to soluble IL-6 receptors [8], then induces dimerization of IL-6 signal transducer gp130 which can activate downstream intracellular signaling cascades. gp130 is a transmembrane glycoprotein that functions as a common receptor for all IL-6-type cytokines [9]. As part of the multiple receptor complexes, gp130 mediates signal transducer and activator of transcription (STAT)-3, mitogen activated protein kinases (MAPK), and phosphatidylinositol 3-kinase (PI3K) signaling pathways, each depends upon different regions of gp130 [10,11]. Until recently, the mechanisms by which LNCaP cells acquire NE properties are not clear yet. Multiple signal pathways, including STAT–3, MAPKs, protein kinase A (PKA), and PI3K dependent signaling pathways, are involved in this IL-6-mediated NE differentiation phenotype [4,6,12,13]. Among these signal pathways, the PI3K pathway is the dominant growth factor-mediated cell survival pathway in LNCaP cells [14]. PI3K protein contains the p85 regulatory subunit and p110 catalytic subunit. IL-6 promotes interaction of p85 subunit of PI3K and IL-6 signal transducer gp130, upregulates tyrosine phosphorylation of p85 and triggers activation of PI3K signaling [15]. Activation of PI3K pathway effector Etk/Bmx (epithelial and endothelial tyrosine kinase) is essential for IL-6-mediated NE differentiation. Overexpression of a dominant-negative Etk can block IL-6 mediated NE differentiation in LNCaP cells [4]. In this study, we have identified androgen as an inhibitor in IL-6-mediated PI3K activation and NE differentiation in LNCaP cells. Blockage of PI3K activation by androgen can inhibit IL-6 induced NE differentiation in LNCaP cells.

MATERIALS AND METHODS

Reagents and Cell Culture

5-dihydrotestosterone (DHT) was obtained from Sigma, LY294002 was purchased from Calbiochem, and hydroxyflutamide (HF) was from Schering. Antibody to PI3K subunit p85α was from Upstate Biotechnology, NSE antibody was from Calbiochem, gp130 antibody was from BD Biosciences Pharmingen, and Bcl-2 antibody was from Santa Cruz Biotechnology. The anti-androgen receptor (AR) polyclonal antibody, NH27, was produced as described [16]. The LNCaP cells were maintained in RPMI-1640 containing penicillin (25 U/ml) and 10% FCS.

Plasmid Construction

The gp130 intracellular C terminal (642–918aa), C1 fragment (642–775aa), and C2 fragment (776–918aa) cDNA were obtained using RT-PCR from LNCaP cDNA library. The cDNAs were then subcloned into pGEX-KG vector to generate GST-gp130C, GST-gp130-C1, and GST-gp130-C2. All GST-fusion plasmids were verified by restriction enzyme analyses and DNA sequencing.

PI3K Kinase Assay

The PI3K activity assay was performed as described by Agazie et al. [17] with modification. After the treatment of DHT, IL-6, and LY294002 (LY) for 20 min, LNCaP cells were harvested and the PI3K complexes were pulled down by PI3K antibody. To measure PI3K activity, a TLC-based assay was employed using phosphatidylinositol 4-5-biphosphate (PIP2) as a substrate. The PI(3,4,5)P3 was quantitated using a Molecular Dynamics PhosphorImager.

Co-Immunoprecipitation (co-IP) and Western Blotting

Immunoprecipitation and Western blotting were performed as described [18]. Briefly, cells were lysed by RIPA buffer (1× PBS, 0.5% Nonidet P-40, 0.5% sodium dexoxycholate, 0.1% SDS, and 1 mM PMSF). Five hundred micrograms of supernatants from cell lysates were incubated with 1.5 μg gp130 polyclonal antibody for 2 hr at 4°C with constant rocking, 25 μl of protein A/G beads were then added into each tube, and incubated for another 2 hr with constant rocking. Anti-Bcl-2 antibody was used as control antibody when doing the experiments. Bcl-2 cannot interact with AR. After four washes with RIPA buffer, the samples were eluted from the beads by boiling in SDS–PAGE sample loading buffer and then resolved by SDS–PAGE gel, and transferred to nitrocellulose membranes. After blotting in TBST (50 mM Tris-HCl, 150 mM NaCl, containing 0.1% Tween-20) with 5% non-fat milk for 1 hr, the membrane was incubated with primary antibody for 1 hr at room temperature. The membrane was then incubated with AP-conjugated second antibodies for another 1 hr at room temperature. The proteins were detected by alkaline phosphate reagents (BIO-RAD).

GST Pull-Down Assay

Expression and purification of GST fusion protein and GST control protein were performed according to the instruction manual (Amersham Pharmacia).

^{35}S-methionine-labeled TNT expressed AR were generated in vitro in TNT-coupled rabbit reticulocyte lysate system (Promega). Equal amounts of purified GST fusion protein bound to glutathione-Sepharose-4B beads were incubated for 2 hr at 4°C with 5 μl of in vitro translated ^{35}S-methionine-labeled AR in the presence or absence of 10 nM ligand, in a total volume of 100 μl of incubation buffer (20 mM HEPES/pH 7.9, 150 mM KCl, 5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.1% Nonidet P-40, 1 mg/ml bovine serum albumin, 10% glycerol, and 1mM PMSF). The beads were washed three times with NENT buffer (20 mM Tris/pH 8.0, 100 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40), boiled in 2× SDS sample buffer, and then analyzed by SDS–PAGE followed by autoradiography.

RESULTS

DHT Blocks IL-6-Mediated PI3K Activation

Androgen can regulate prostate cancer cell proliferation and differentiation. However, the mechanism by which androgen regulates cell differentiation is largely unknown. LNCaP cell NE differentiation induced by IL-6 is a well-studied cell model to study differentiation. PI3K-Etk signaling activation is crucial in this event [4]. We, and others [19], have observed that DHT is linked to both PI3K activity and NE differentiation in LNCaP cells. Therefore, we first asked if DHT could directly affect IL-6-mediated PI3K activation. Serum-free medium was used to deprive LNCaP cells of androgenic support and growth factors for the remaining studies. We assessed the level of PI3K activity directly using PI3K kinase assay under the treatment of DHT, IL-6, or DHT plus IL-6. The PI3K inhibitor LY294002 served as a positive control to block IL-6-mediated PI3K activation. As shown in (Fig. 1), IL-6 induced PI3K activity in a short time. Surprisingly, DHT was able to block IL-6-mediated PI3K activation to a background level within 20 min. While we do not rule out AR genomic function, it seems androgen and AR are able to block IL-6-induced PI3K activity through non-genomic activity. This is reasonable since earlier reports show that other steroid receptors can activate the PI3K/Akt signaling pathway via non-genomic actions. For example, 17β-estradiol can activate PI3K through non-genomic actions of estrogen receptor in endothelial cells [20].

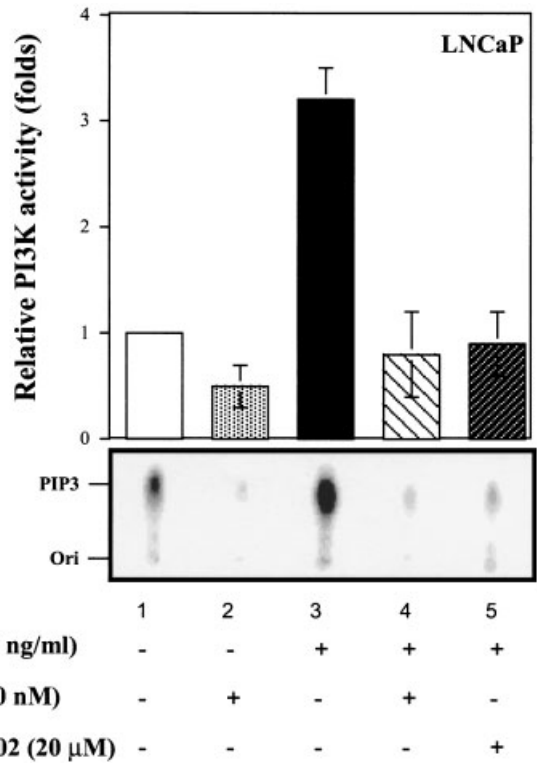

Fig. 1. Androgen blocks IL-6-mediated PI3K activation in LNCaP cell lines. LNCaP cells were cultured under the indicated conditions. After the treatment of 5α-dihydrotestosterone (DHT), IL-6, and LY294002 (LY) for 20 min, respectively, cells were harvested and/or PI3K complexes were pulled down by PI3K antibody. PI3K activity assays were performed. The PIP3 signals were quantitated by PhosphorImager, and the relative changes of PI3K activity are shown. The data are representative of three independent experiments with similar findings.

DHT Is Involved in the IL-6-Induced NE Differentiation of LNCaP Cells

Next, we examined DHT/AR effects on IL-6-induced LNCaP cell morphology change. LNCaP cells were serum starved and then treated with various reagents for 2 days. Consistent with previous reports [4,19], IL-6 (25 ng/ml) treatment induced NE differentiation in LNCaP cells (Fig. 2B). As expected, DHT was able to block NE differentiation in LNCaP cells. Further study showed HF, an antiandrogen, can counteract the DHT effect on IL-6-induced NE differentiation (Fig. 2C,D). HF alone is a weak agonist of the mutant AR in LNCaP cells. However, we and others have observed that HF can reduce or block other DHT effects, such as DHT effects on AR transcriptional activity [21] and apoptosis [22] of LNCaP cells, presumably through their competition to bind to AR. HF alone under our conditions does not induce NE differentiation (Fig. 2F). These observations suggest AR is involved in this event. We also tested the NE differentiation marker NSE by Western blot (Fig. 2G). IL-6 treatment increased NSE expression level, consistent with previous reports [4,19]. Treatment of DHT and IL-6 together was able to decrease NSE expression level compared to IL-6 treatment alone. HF can reverse the DHT effect on IL-6-induced NE differentiation.

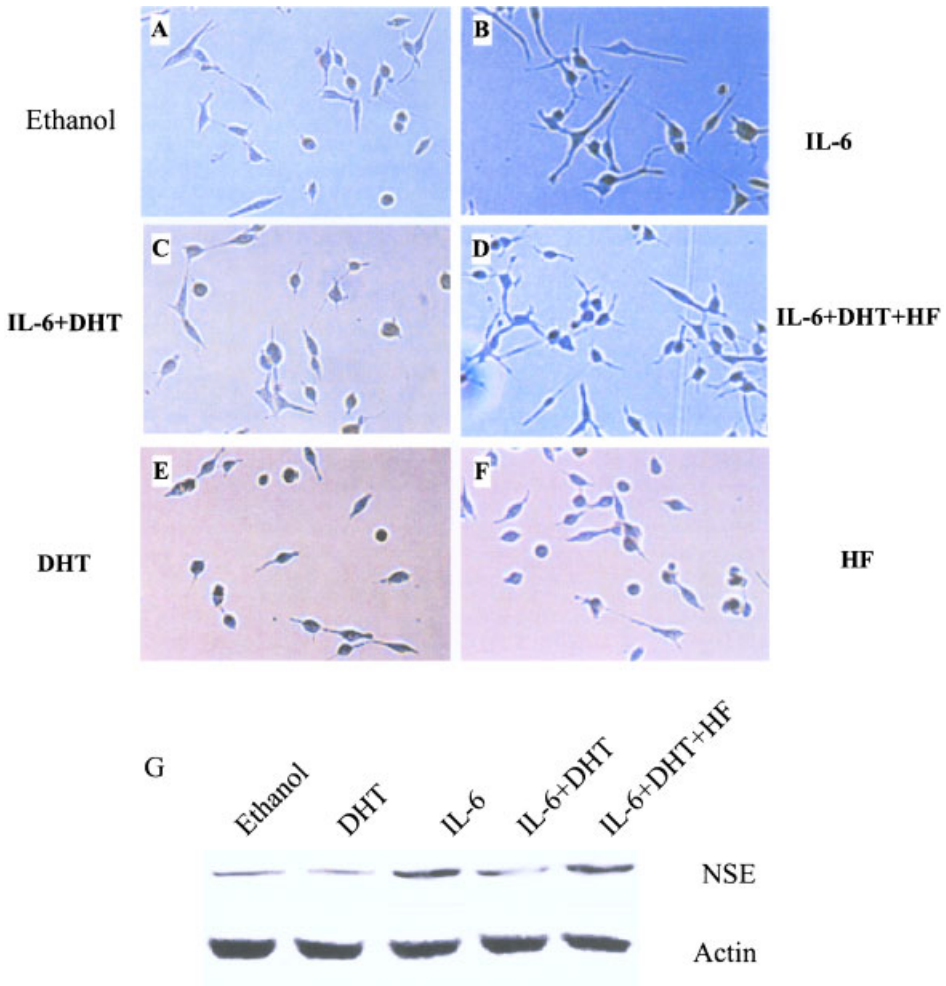

Fig. 2. DHT and androgen receptor (AR) are involved in the IL-6-induced NE-like differentiation of LNCaP cells. **A**–**F**: Morphologies of LNCaP treated with indicated drugs. (A, B) IL-6 induced LNCaP NE-like differentiation. A: LNCaP cells in the serum-free medium; (B) LNCaP cells treated with 25 ng/ml IL-6 for 48 hr. (C, D) DHT treatment blocks the IL-6-induced morphological changes, whereas HF, an anti-androgen reversed its effect. C: LNCaP cells treated with 25 ng/ml IL-6 plus 10 nm DHT for 48 hr; (D) LNCaP cells treated with 10 nM DHT, 5 μM HF, and 25 ng/ml IL-6 for 48 hr. For comparison, LNCaP cells were treated with ethanol (A), 10 nM DHT (E) or 5 μM HF (F). **G**: NSE expression in LNCaP after indicated treatment. Actin was used as loading equal marker.

Those changes in NSE protein expression correlated with the cell morphology changes (Fig. 2G). These findings suggest that androgen goes through AR to block IL-6-induced NE differentiation in LNCaP cells.

DHT Promotes Interaction Between AR and gp130 While Decreasing the Complex Formation Between gp130 and PI3K

We then examined if rapid AR action is able to affect the interaction between gp130 and PI3K, a step required for IL-6-mediated PI3K activation and subsequent NE differentiation [15,23]. It is still unknown how IL-6 activates the PI3K pathway. In response to IL-6, Gab1, a scaffolding adapter protein, is tyrosine phosphorylated and interacts subsequently with SHP2 and PI3K. Gab1 contains binding sites for Grb2, SHP2, and PI3K [9,23,24]. IL-6 promotes coprecipitation of p85 with gp130, a process when interrupted will lead to blockage of PI3K activation [15]. We therefore hypothesize that AR may directly block gp130–p85 complex formation. A series of co-IP experiments were carried out. We used anti-Bcl-2 antibody as an irrelevant control antibody when doing the experiments. Bcl-2 cannot interact with AR and anti-Bcl-2 antibody did not pull down AR and p85 (data not shown). As shown in Figure 3A, treatment with IL-6 enhanced the complex formation between gp130 and PI3K (lane 4 vs. lane 2). In addition to IL-6 treatment, androgen within 20 min significantly decreased the complex formation between gp130 and PI3K (lane 5 vs. lane 4). At the same time we also found that androgen markedly enhanced

AR–gp130 complex formation (lane 5 vs. lane 4 and lane 3 vs. lane 2). It is possible that AR-gp130 interaction may result in the interruption of the formation of PI3K and gp130, presumably due to an overlapping binding site in the gp130 complex. To further support this hypothesis, we tested whether AR can directly interact with gp130 in vitro. We performed a GST pull-down assay using TNT expressed AR and GST-gp130 intracellular fragment (642–918aa) fusion proteins as described in the methods. As shown in (Fig. 3B), recombinant GST-gp130 intracellular domain was found to bind to in vitro translated ^{35}S-methionine-labeled AR in the presence or absence of DHT (lanes 3, 4, 7, 8). Moreover, androgen seemed to enhance this interaction consistent with above co-IP results. Further mapping studies showed that the binding domain interacting with AR was in the C-terminal amino acid 776–918 of gp130 (lanes 7, 8). The data indicate that AR may play a role in IL-6-mediated gp130 signaling. In summary, we have identified DHT as an inhibitor in IL-6-mediated PI3K activation and NE differentiation in LNCaP cells. We further demonstrate that AR plays an important role in this event by directly interacting with the IL-6/PI3K signaling component gp130. The interaction between AR and gp130 may account for the decreased complex formation between gp130 and PI3K, resulting in the inhibition of PI3K activation and NE differentiation of LNCaP cells. A simplified model, which does not include many proteins in the IL6/gp130 signaling pathways, is shown in (Fig. 4). This model, however, does not rule out the possibility that AR may also directly interact with other proteins in the gp130 complex in the IL-6-PI3K signaling pathway. Therefore, further studies may yield more detailed mechanisms of how androgen influences the NE differentiation.

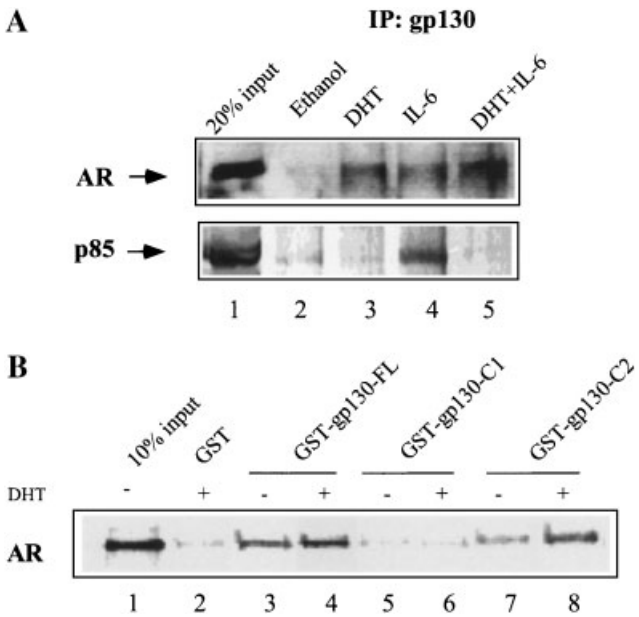

Fig. 3. AR interacts with gpl30. **A**: In vivo interaction of AR and gpl30. Co-immunoprecipitation was performed with monoclonal gpl30 antibody using equal amount of lysates prepared from LNCaP cells treated with different reagents as indicated in the legend. The co-immunoprecipitates were analyzed by Western blots with AR or p85 antibody. The results were representative of three independent experiments. **B**: In vitro interaction between gpl30 and AR. The following GST fusion proteins were used: GST-gpl30-C (642–918 aa), GST-gpl30-Cl (642–775 aa), and GST-gpl30-C2 (776-918 aa). GST pull-down assay was used with in vitro translated ^{35}S-labeled AR and purified GST- or GST fusion protein-bound glutathione-sepharose beads in the absence (–) or presence (+) of 10 nM DHT. The input represents 10% of the amount of labeled protein used in the pull-down assay. The samples were subjected to SDS–PAGE and autoradiography. The results were representative of three independent experiments.

DISCUSSION

Androgen has been shown to regulate cell proliferation and differentiation in many cells, including normal and prostate cancer cells [25–27]. Normally, steroid receptors exert their action by acting as ligand-inducible transcriptional factors. However, this classical model of steroid transcriptional action cannot explain some steroid actions, including regulation of cell proliferation and differentiation [28]. Alternatively, steroids interact with their cognate receptors to induce rapid intracellular signaling [29,30]. To modulate cell differentiation and proliferation, estradiol activates the signal transduction pathway Src/Ras/Erks in human mammary cancer derived cell line MCF-7 within minutes [31]; synthetic androgen R1881 stimulates the Src/Raf-1/Erks signaling pathway in LNCaP cells where AR binds to the membrane-bound SH3 domain of Src through a proline-rich stretch to trigger the signaling [32]. Thus, it is suggested that steroid

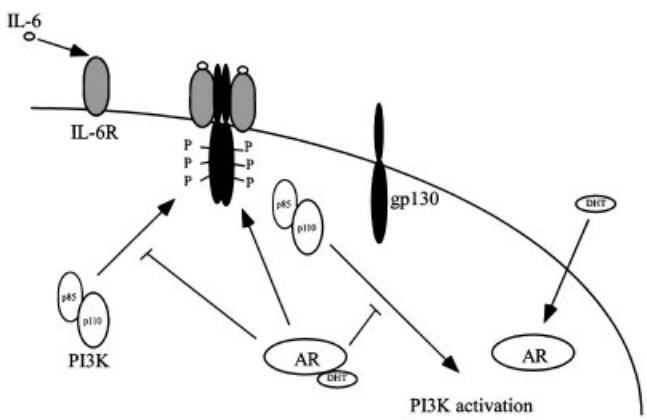

Fig. 4. A simplified model of DHT/AR action in the IL-6-mediated PI3K pathway and NE-like differentiation.

receptors act outside the nucleus, and play important roles via non-genomic approach.

IL-6 is a multi-functional cytokine that can regulate prostate cancer cell growth and AR activity. How IL-6 signaling interacts with androgen signaling in prostate cancer cells in vitro is well documented [2]. In our previous study, we found IL-6 could regulate AR activity and prostate cancer cell growth via three different signal pathways, STAT3, MAPK, and PI3K-Akt. Different signal pathways have different effect on AR activity. For example, IL-6 can increase AR activity via either the STAT3 or MAPK pathways, whereas IL-6 suppresses AR activity via the PI3K-Akt pathway. Therefore, the overall effect of IL-6 on AR activity is determined by the balance of these various pathways [33]. IL-6 also can induce LNCaP cells NE differentiation via various signal pathways, including STAT3, MAPK, PKA, and PI3K [4,6,12,13]. PI3K/Akt pathway plays a crucial role in cell differentiation in several cell lines. For example, retinoic acid-mediated neural differentiation of SH-SY5Y requires PI3K activation [34]. The PI3K-Etk signaling activation is crucial for the IL-6-mediated LNCaP cell NE differentiation [4,18]. Interestingly, we [35] and others [19] observed that androgen depletion can induce both LNCaP cell NE differentiation and PI3K activation. In the present study, we found DHT can inhibit PI3K activity. Thus, we assumed that AR signaling may affect IL-6-mediated NE differentiation by blocking the PI3K activity. Here, our data suggest AR can directly interact with gp130, resulting in the blockage of protein complex formation between p85 and gp130, therefore inhibiting IL-6 induced PI3K activation.

Interaction between the IL-6 signaling and androgen signaling has been well documented. However, most studies focused on how IL-6 signaling affects AR signaling. Here, we report androgen and AR may play an inhibitory role in IL-6 signaling. During the time this article was under preparation, another article was published on repression of NE differentiation by AR in LNCaP cells [36]. AR silencing by AR siRNA can induce NE differentiation in LNCaP cells. This data is consistent with our hypothesis that AR is directly involved in LNCaP cells NE differentiation. However, the authors did not provide mechanisms how AR represses the LNCaP cells NE differentiation. AR may repress NE differentiation via either genomic effect or non-genomic effect, or both. Our finding supports that AR may go through a non-genomic effect to block IL-6-induced LNCaP cell NE differentiation, although we do not rule out the possibility that AR may also repress NE differentiation via genomic effects. In fact, we expect an integration of genomic and non-genomic actions of androgen receptors to regulate prostate cancer cell survival and differentiation.

CONCLUSIONS

Here, we demonstrate that androgen can block IL-6-mediated PI3K activation within minutes and block NE differentiation in LNCaP cells. PI3K activation is required for IL-6-mediated NE differentiation in LNCaP cells. Our results suggest that AR can directly interact with gp130. Treatment of androgen enhances the interaction between AR and gp130, inhibits gp130-PI3K protein complex formation, and subsequently blocks PI3K activation, resulting in the blockage of NE differentiation induced by IL-6 in LNCaP cells. Taken together, these data support a non-transcriptional role for AR in regulating prostate cancer cells differentiation.

ACKNOWLEDGMENTS

We thank Eungseok Kim, Yueh-Chiang Hu, and other laboratory members for technical assistance; and Karen Wolf for help in preparation of the article.

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