RESEARCH ARTICLE

The co-regulators SRC-1 and SMRT are involved in interleukin-6-induced androgen receptor activation

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ABSTRACT. ***Background:*** **The androgen receptor (AR) can be stimulated by interleukin-6 (IL-6) in the absence of androgens to induce prostate cancer progression. The purpose of this study was to investigate whether the co-activator steroid receptor coactivator-1 (SRC-1) and co-repressor silencing mediator for retinoid and thyroid hormone receptors (SMRT) are involved in IL-6-induced AR activation.** ***Methods:*** **The effects of IL-6 on LNCaP cell proliferation were monitored using real-time cell analysis (RTCA) iCELLigence system. The impacts of IL-6 on the association of the AR with SRC-1 and SMRT were investigated using the mammalian two-hybrid assay.** ***Results:*** **IL-6 increased the proliferation of LNCaP cells with maximal induction at 50 ng/mL. The AR-SRC-1interaction was enhanced by IL-6, with maximal induction at the concentration of 50 ng/mL ($P<0.05$). IL-6 decreased the AR-SMRT interaction and a marked reduction was detected at 50 ng/mL ($P<0.05$).** ***Conclusions:*** **IL-6 enhances LNCaP cells proliferation, which suggests that IL-6 might cause AR-positive prostate cancer growth through activation of the AR. The mechanism of IL-6-induced AR activation is mediated through enhancing AR-SRC-1 interaction and inhibiting AR-SMRT interaction. We have shown a significant role for SRC-1 and SMRT in modulating IL-6-induced AR transactivation.**

Key words: steroid receptor coactivator-1, silencing mediator for retinoid and thyroid hormone receptors, androgen receptor, interleukin-6

The androgen receptor (AR) is an androgen-dependent transcription factor that belongs to the superfamily of nuclear receptors [1-3]. The endogenous androgenic ligands bind to the AR, which induces a conformational change in the AR and leads to the shuttling of the receptor from the cytoplasm to the nucleus. Following translocation to the nucleus, the ligand-AR complex binds to the specific androgen response elements (AREs) in the promoters of androgen-regulated genes, which facilitates the activation of androgen-targeted genes such as prostate-specific antigen [4].

The AR is believed to play critical roles in the development and progression of prostate cancer [5, 6]. Therefore, when initially identified, most prostate cancers require androgenic stimulation for growth. After androgen withdrawal, the prostate cells undergo an active process of programmed cell death. However, after an initial response to androgen deprivation therapy, prostate cancer usually recurs in a form that grows independent of androgen and is no longer treatable [7]. Accumulating evidence suggests that the androgen-independent phenotype results when prostate cells acquire a growth mechanism in a paracrine and autocrine manner through production of growth factors and cytokines [8, 9]. One of these cytokines is interleukin-6 (IL-6) which regulates prostate cancer growth by activating AR-dependent gene expression in the absence of androgen [7, 10]. IL-6 is a glycoprotein consisting of 212 amino acids encoded by the IL-6 gene [11, 12]. Accumulating clinical observations have revealed the frequent association of elevated serum IL-6 levels with androgen-independent prostate tumors [13, 14]. IL-6 is involved in androgen-independent progression of prostate cancer. Binding of IL-6 to its receptor leads to activation of Janus kinase-signal transducer and activator of transcription (JAK/STAT), mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase/Akt kinase (PI3-K/AKT) pathways [15, 16]. The signaling pathways activated by IL-6 have been reported to mediate signaling cross-talk with the AR, which may play an important role in prostate cancer progression [17, 18].

The underlying molecular mechanisms of IL-6-induced AR activation remain unidentified. One proposed mechanism may involve the action of co-regulatory proteins of the AR [19]. Co-regulators are defined as proteins that interact with nuclear receptors to regulate transactivation of target genes, which include co-activators enhancing transactivation and co-repressors reducing expression of target genes [20, 21]. Co-regulators are known to influence AR transcription by recruiting to the promoter through protein-protein interactions with AR to facilitate DNA occupancy,

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chromatin remodeling, or the recruitment of general transcription factors associated with the RNA polymerase II holocomplex [2].

Steroid receptor coactivator-1 (SRC-1) was the first co-activator to be discovered and belongs to a larger p160 family of nuclear receptor co-activators [22]. SRC-1 interacts with the AR N-terminal domain (NTD) and thereby promotes AR dimerization and activation in the presence of androgens. SRC-1 expression is also increased in androgen-independent prostate cancer to stimulate AR activity [23, 24]. One study demonstrated that IL-6-mediated functional activation of the AR involved the activation of SRC-1 and promoted AR-SRC-1 association [25]. However, another study showed that IL-6 did not affect p160 co-activators such as SRC-1 recruitment to the transcription initiation complex on the PSA enhancer and promoter [26]. In our previous study, we have shown that the AR interacts with SRC-1 in the absence of androgen [27]. Therefore, SRC-1 may be recruited by the AR upon ARE of androgen-targeted genes and subsequently function to enhance AR transcription. We speculate SRC-1 is a key facilitator of transactivation of the AR mediated by IL-6.

In addition to the mechanisms associated with co-activators, recruitment of co-repressors also plays important role in AR activity. The data have shown that co-repressors associate with the AR and repress its transcriptional activity [28]. The co-repressor silencing mediator for retinoid and thyroid hormone receptors (SMRT) has been shown to interact with both the unliganded and liganded forms of AR to play key roles in the transcription of AR [29]. However, in another study, the data have shown that IL-6 did not lead to the recruitment of SMRT to the transcription initiation complex on the PSA enhancer and promoter [26]. Our previous study has shown that SMRT is recruited to the AR and interacts with it in the absence of dihydrotestosterone (DHT) [30]. We hypothesized that the association between the AR and SMRT might be an underlying mechanism of IL-6-dependent AR regulation.

In this study, we investigated whether IL-6 induces AR-positive cell proliferation without treatment with androgens. We have demonstrated that IL-6 induces proliferation of LNCaP human prostate cancer cells using real-time cell analysis (RTCA) iCELLigence system. Furthermore, we studied whether the mechanism of androgen-independent activation of the AR by IL-6 involved the co-regulatory proteins. We provide evidence to suggest that IL-6 induces AR activation by enhancing the associations of the AR with SRC-1 and reducing the recruitment of SMRT to the AR. These findings reveal a new insight into the IL-6- mediated transactivation of the AR, which might play an important role in androgen-independent prostate cancer progression.

METHODS AND MATERIALS

Reagents

Human interleukin-6 (IL-6) was purchased from Pepro-Tech Inc (Rocky Hill, USA). The purity of the chemical was greater than 98%. A stock solution of IL-6 was prepared in phosphate-buffered saline (PBS) at a concentration of 100 μg/mL, stored at -20 °C, and diluted to desired concentrations in phenol red-free RPMI1640 medium (Life Technologies, Carlsbad, California, USA) before use. The ESCORT V Transfection Reagent was from Sigma Chemical Co (St. Louis, MO, USA), and the chloramphenicol acetyl transferase enzyme-linked immunosorbent assay (CAT-ELISA) kit was from Roche Molecular Bioch (Mannheim, Germany). The Bicinchoninic Acid (BCA) Protein Assay kit was obtained from Beyotime Institute of Biotechnology (Shanghai, China).

Plasmids and plasmid construction

The plasmids used in this study had been constructed previously. The Mammalian Matchmaker^{TM} Two-Hybrid Assay Kit was purchased from Clontech Laboratories, Inc. (Palo Alto, CA, USA). The pG5CAT was used as the reporter vector, which contains the CAT gene downstream of five consensus GAL4 binding sites and the minimal promoter of the adenovirus E1b gene. The fusion vector pVP16-ARNTD was constructed by fusing the gene of AR N-terminal regions encoding 1-660 amino acid residues to the VP16 activation-domain (AD) in the pVP16 vector. The fusion vector pM-ARLBD was constructed by fusing the gene of AR C-terminal regions encoding 624-919 amino acid residues to the GAL4 DNA binding domain (DBD) in the pM vector. The fusion vector pM-SRC-1 was generated by fusing the C-terminal domain encoding 989-1240 amino acid residues of SRC-1 to the GAL4 DNA-binding domain (DNA-BD) in the pM vector. The fusion vector pM-SMRT was generated by fusing the C-terminal domains encoding 2101-2400 amino acids of SMRT to the pM vector.

Cell culture

The African monkey kidney cell line CV-1 and the LNCaP prostate cancer cell line were purchased from Institute of Biochemistry and Cell Biology in Shanghai, Chinese Academy of Science (Shanghai, China). The cells were maintained in phenol red-free RPMI1640 medium supplemented with 10% charcoal-dextran-stripped FBS (CDS-FBS) at 37°C, in an atmosphere of 5% CO_2/air.

Real-time cell analysis (RTCA) iCELLigence system

The RTCA iCELLigence system (ACEA Biosciences Inc., San Diego, CA92121) is comprised of three components including an electronic sensor analyzer, a device station, and two 8-well strips. The RTCA iCELLigence system based on electrical impedance measurement is a label-free and simple homogeneous assay system for cell-based assays. Briefly, 150 μL of medium (RPMI1640) was added to an eight-well E-plate and background readings were recorded. LNCaP cell suspension at cell density of48000 cells/well, in a total volume of 300 μL, was added to each well of the E-plate. The cell proliferation rates were recorded at 15 minutes intervals. After 24 h, different concentrations of IL-6 were added to the cultures and continuously monitored every 15 minutes for up to 110 h.

Mammalian two-hybrid interaction assays

The mammalian two-hybrid assay is based on the ability of a protein fused to the Gal4 transcription factor DBD in the pM vector to recruit a protein linked to a VP16 AD

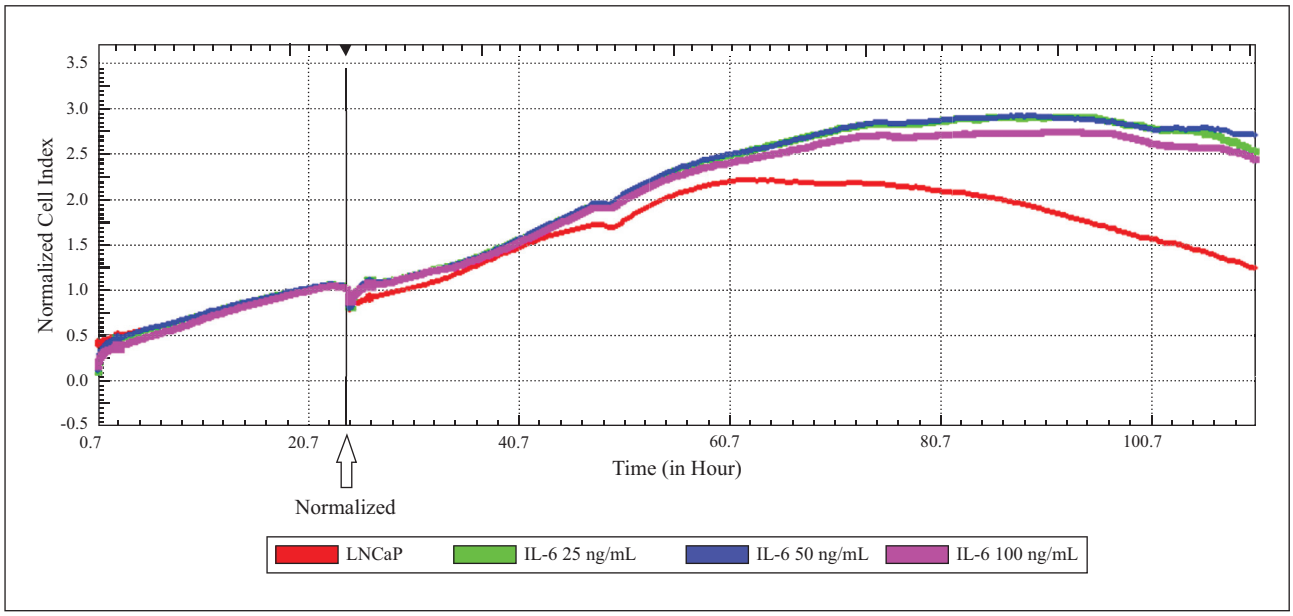

Figure 1

Effects of IL-6 on the proliferation of LNCaP cells. LNCaP cells were incubated for 24 h with medium, then stimulated with 0, 25, 50, 100 ng/ml of IL-6. The proliferation was measured over time using the RTCA iCELLigence system. Cell indexes were recorded at 15 minutes intervals. Each curve at each concentration was an average of two replicates. The results showed that IL-6 can increase the proliferation of LNCaP cells without ligand.

in the pVP16 vector to activate transcription of pG5CAT reporter vector. To test for the protein-protein interactions, the previously constructed vectors were used. The CV-1 cells, which do not have endogenous AR, the background value being low, were plated in triplicate in six-well microtiter plates (Corning Incorporated, USA), at a density of about 1.5×10^6 cells per well with 2 mL RPMI1640 medium containing 10% CDS-FBS and left to incubate for 24 h. The cells were co-transfected with the reporter vector pG5CAT, pVP16-ARNTD or pM-ARLBD, pM-SRC-1 or pM-SMRT using the ESCORT V transfection reagent. Then the transfection medium was replaced with medium containing various concentrations of IL-6. The cells were harvested 24 h after dosing. After rinsing three times with PBS, the cells were lysed with 1×lysis buffer (Promega, Madison, WI, USA, 400 μL/well). After centrifugation at 12,000 × g for five min to remove debris, the cell lysates were either analyzed immediately or quick-frozen at -80°C. CAT was measured with the commercial CAT-ELISA kit following the manufacturer's instructions. The values were normalized for cellular protein, determined by the BCA Protein Assay kit. The relative CAT amounts were presented as -fold induction, which is calculated relative to the untreated control vector.

Statistical analysis

Data for the measurement of the cell proliferation rate are presented in the form of a unitless variable termed the Cell Index (CI). The CI was derived to represent cell status based on the electrical impedance measured. The CI at each time point was defined as (Rn-Rb)/15, where Rn is the cell-electrode impedance of the well and the Rb is the background impedance of the well with medium alone. Data from the various treatments were normalized at the time point when the IL-6 was added so that the mode of action of IL-6 could be determined. In the mammalian two-hybrid assay, the values were expressed as mean ± S.D. for three separate experiments with triplicate wells, for each treatment dose. ANOVA was used for multiple comparisons, followed by Dunnett's t-test for multiple comparisons with controls. The level of significance was set at $P<0.05$.

RESULTS

Proliferation stimulation effect of IL-6 on LNCaP cells

The data have shown that IL-6 can function as a proliferation factor for human LNCaP prostate cancer cells [12]. The RTCA iCELLigence system was used to monitor the effects of different concentrations of IL-6 (0, 25, 50, 100 ng/mL) on the proliferation of LNCaP cells dynamically. The results indicated the stimulatory effect of IL-6 on cell proliferation in LNCaP cells, with maximal induction at the concentration of 50 ng/mL (*figure 1*). It is suggested that IL-6 can enhance the proliferation of LNCaP cells in the absence of androgen *in vitro*.

IL-6 cannot induce the interactions of AR NH_2/COOH

In order to investigate the mechanism of IL-6-induced AR signaling, we investigate the interaction of IL-6-induced AR NH_2/COOH with the mammalian two-hybrid assay. In this assays, the CV-1 cells were co-transfected with the reporter vector pG5CAT, pVP16-ARNTD and pM-ARLBD. The cells were treated with IL-6 at the indicated concentrations and then tested for the expression of CAT reporter. We failed to detect CAT reporter expression in the mammalian two-hybrid assay (data not shown). This finding indicates that the AR NH_2/COOH interaction may not be involved in the cell proliferation induced by IL-6.

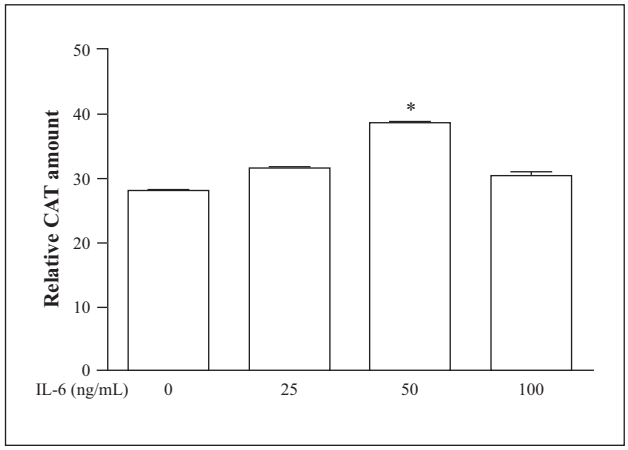

Figure 2
Effects of IL-6 on the interactions between AR/SRC-1. In the mammalian two-hybrid assay, CV-1 cells were co-transfected with the reporter vector pG5CAT, pVP16-NH_2, pM-SRC-1. Cells were treated with IL-6 at concentrations of 0, 25, 50, 100 ng/mL and then the expression of CAT reporter was tested. The data showed that IL-6 increased the interaction between the AR and SRC-1, with the maximal induction at the concentration of 50 ng/ml ($P<0.05$). Data are expressed as mean ± S.D.

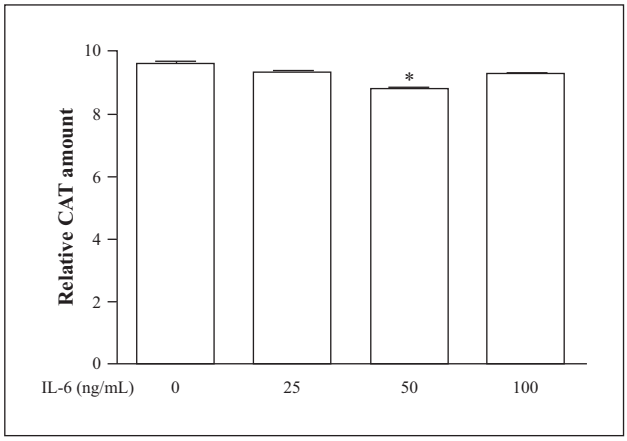

Figure 3
Effects of IL-6 on the interactions between AR/SMRT. In the mammalian two-hybrid assays, the CV-1 cells were co-transfected with the reporter vector pG5CAT, pVP16-NH_2, pM-SMRT. The cells were treated with IL-6 at concentrations of 0, 25, 50, 100 ng/mL and then the expression of CAT reporter was tested. The results indicated that IL-6 decreased the interaction between the AR and SMRT, and a marked reduction was detected at the concentration of 50 ng/mL ($P<0.05$). Data are expressed as mean ± S.D.

IL-6 can enhance the interaction between the AR and co-activator SRC-1.

To study the interactions of AR/SRC-1 mediated by IL-6, the mammalian two-hybrid assay was used. We co-transfected the reporter vector pG5CAT, pVP16-ARNTD, pM-SRC-1 to CV-1 cells, stimulated the cells with different concentrations of IL-6, and then tested the expression of CAT reporter. The data showed that the interaction between the AR NTD and SRC-1 was enhanced by IL-6, with the maximal induction at the concentration of 50 ng/mL ($P<0.05$) (*figure 2*). The results suggests that IL-6 can increase the interaction between AR and SRC-1, which is in accordance with the previously published result showing interaction of AR with SRC-1 in the presence of IL-6 [25].

IL-6 can suppress the interaction between the AR and co-repressor SMRT.

The mammalian two-hybrid assay was performed to study whether the interaction of the AR with SMRT was mediated by IL-6. In the study, the reporter vectors pG5CAT, pVP16-ARNTD, pM-SMRT were co-transfected into CV-1 cells. Different concentrations of IL-6 were added to the cells and then the expressions of CAT reporter were tested. The results indicated that IL-6 decreased the interaction between the AR and SMRT and a marked reduction was detected at the concentration of 50 ng/mL ($P<0.05$) (*figure 3*). The result substantiates the idea that the recruitment of SMRT to the AR is inhibited by IL-6. The suppression of the interaction between the AR and SMRT relieves the repression function of SMRT, and then AR activation is stimulated by IL-6.

DISCUSSION

In the present study, we have applied the iCELLigence RTCA system to monitor cell proliferation real-time by measuring changes in electric impedance between two gold electrodes embedded in the bottom of the cell culture wells [31, 32]. We provide experimental evidence that IL-6 plays an important role in the induction of proliferation of LNCaP cells. The association of the AR with SRC-1 is shown to be enhanced by IL-6. In addition, we demonstrate that IL-6 represses the interaction between the AR and SMRT. Our results showed that IL-6 modulates AR action through the differential recruitment of cofactors to the AR.

It has been demonstrated that IL-6 can act as a growth factor for both normal primary prostate epithelial cells and LNCaP prostate cancer cells [7, 33]. In the present study, using the novel iCELLigence system, we found that IL-6 increases human LNCaP prostate cancer cell proliferation, which is consistent with previous reports. We confirm the role of IL-6 in the promotion of proliferation of these cells in the absence of androgens. LNCaP cells express functional, but mutant AR, therefore, IL-6 could activate AR transactivation in an androgen-independent manner in LNCaP cells [12]. AR activation by IL-6 was first described by Hobisch *et al*. in 1998 [10], and similar results were confirmed by other investigators [16, 34]. It is suggested that crosstalk between IL-6 and AR may have an important effect on the activation of AR signaling. Even though there are likely to be other, unknown ligands involved in the mechanism of LNCaP cell proliferation, the potentiation effects of IL-6 on AR transactivation and prostate cancer growth is undeniable.

It has become clear that the transcriptional activity of the AR is modulated by co-activators [19]. The recruitment of co-activators has been suggested to be a possible mechanism underlying in the activation of the AR by IL-6. However, previously published data have yielded conflicting results regarding the effect of co-activators on AR activation by IL-6, with studies showing positive findings, and others showing negative findings [25, 26]. The present study investigates whether the AR-SRC-1 interaction is involved in the AR activation induced by IL-6. We examined the effects of IL-6 on interaction between the AR and SRC-1 using the mammalian two-hybrid assay. The

results have shown that AR NTD interacts with SRC-1 in the absence of DHT, and the interaction between AR and SRC-1 is enhanced by IL-6. It is suggested that the AR activation induced by IL-6 is due to SRC-1 recruitment to the AR. In the cell nucleus, SRC-1 is recruited to the potential binding sites determined on the AR NTD in the present of IL-6. Then, SRC-1 may activate the AR in the absence of its cognate ligand. The association of the AR with SRC-1 potentiates AR activity via recruitment of CBP/p300, a histone acetyltransferase. The process results in an up-regulation of the target gene transcription, inducing the implementation of the biological response related to the AR. Therefore, the interaction between the AR and SRC-1 is involved in AR transcriptional function induced by IL-6. It has been reported that the elevated levels of SRC-1 in tumor cells is accompanied by enhanced levels of IL-6 in the blood of patients with androgen-independent prostate cancer [23, 24], thus SRC-1 plays an important role in the AR activation by IL-6. The data provide a rationale for the relationship between elevated expression of SRC-1 and activation of the AR by IL-6. One previous study has shown that protein-protein interaction between AR and SRC-1 is dependent upon IL-6 [25]. Taken together, although another study has not found SRC-1 recruitment involved in the effects of IL-6 on the AR, we strongly suggest an important role for SRC-1 in the mechanism of transactivation of the AR induced by IL-6. IL-6 can enhance AR transactivation via AR-SRC-1 interaction to modulate AR-targeted gene expression in the absence of ectogenous androgens.

The AR also binds to co-repressors, which indicates that co-repressors might contribute to AR function [19, 35, 36]. SMRT has been shown to interact with the AR in the absence of androgen [29, 30]. However, the effects of IL-6 on interaction between AR and SMRT are unknown at the present time. We have detected the interaction of AR with SMRT in the presence of IL-6 through the mammalian two-hybrid assay. The results have demonstrated the AR NTD bond to SMRT in the absence of AR ligand DHT, and the interaction between AR and SMRT is inhibited by IL-6. SMRT acts as a negative regulator of AR activity contributing to the inhibitory functions of AR. Therefore, a reduced AR-SMRT interaction would decrease the repressive effect and might subsequently lead to transactivation of the AR. We speculate that the reduced recruitment of SMRT would decrease the interaction of SMRT with histone deacetylases to increase transcription. It could be shown that the reduced recruitment of SMRT plays an important role in AR activation and accounts for elevated AR activity induced by IL-6. Therefore the inhibition of AR-SMRT interaction is required for AR activation in response to IL-6.

The recruitment of co-activators and co-repressors to the AR is involved in the regulation AR transcription [37, 38]. It is suggested that the interaction of the AR with co-activators and co-repressors could potentially play important roles in modulating both agonist and antagonist-regulated AR function [36, 39]. Therefore, both types of co-regulators may be involved in AR-target gene activation and in repression, which may be dependent upon the relative levels of the co-activators *versus* co-repressors. Alterations in the balance of co-activator- to co-repressor-binding to the AR have been shown to influence AR activation. The ratios of co-activators and co-repressors bound to the AR might determine whether the outcome is stimulatory or inhibitory [40]. The increased AR transcription activity correlates with increased recruitment of co-activators such as SRC-1 to the AR, while antagonists may recruit more co-repressors such as SMRT to the AR [39]. In the present study, we found that the AR-SRC-1 interaction is enhanced, while the AR-SRMT interaction is decreased in the presence of IL-6. We predict that IL-6 stimulates the recruitment of SRC-1 to the AR, while the recruitment of SMRT will be inhibited by IL-6. The effects on the interactions of the AR with SRC-1 and SMRT contribute to the AR activation induced by IL-6. We have shown that SMRT interacts with the AR through a binding site that overlaps the SRC-1 site in the AR NTD, which is consistent with previous studies [39]. Co-activators and co-repressors may compete with each other for binding to AR. It is reasonable to speculate SRC-1 can compete with SMRT for binding to the AR NTD in the presence of IL-6. It is possible that the AR is able to recruit more SRC-1.The enhancement of AR-SRC-1 interaction will repress the association of the AR with SMRT. The balance of the recruitment of SRC-1 and SMRT may then determine the overall effect of IL-6 on AR transactivation. Therefore, the increase in SRC-1 recruitment, along with the decrease in SMRT recruitment, will account for the AR activation mediated by IL-6.

In summary, we have shown that IL-6 stimulates LNCaP cell proliferation in the absence of androgens. The co-regulators SRC-1 and SMRT may contribute to IL-6-induced AR transactivation and cell proliferation. We have shown that IL-6 enhances the interaction between the AR NTD and SRC-1, while it inhibits the interaction between the AR NTD and SMRT. Therefore, the IL-6-induced AR transactivation results from enhancing recruitment of SRC-1 and inhibiting recruitment of SMRT. Our study provides the new insight into the role of SRC-1 and SMRT in IL-6-induced AR activation. The mechanism dependent upon SRC-1 and SMRT may help to elucidate the events involved in the progression of androgen-independence prostate cancer, and the biological and clinical significance of the AR activation by IL-6.

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