

Interleukin-4 Activates Androgen Receptor Through CBP/p300

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BACKGROUND. Aberrant activation of androgen receptor (AR) plays an important role in the progression of castration resistant prostate cancer. Interleukin-4 (IL-4) enhances AR activation in the absence of androgen and stimulates castration resistant growth of androgen-sensitive prostate cancer cells. However, the mechanism of IL-4 mediated AR activation has not yet been revealed.

METHODS. The effect of IL-4 on CBP/p300 expression was examined by Western blot analysis. The effect of IL-4 on the interactions of AR and CBP/p300 was examined by co-immunoprecipitation and ChIP assays. CBP/p300 siRNA was used to knockdown CBP/p300 expression to examine the role of CBP/p300 expression on IL-4 mediated AR activation.

RESULTS. We found that IL-4 increases CBP/p300 protein expression and enhances interaction of AR with CBP/p300 proteins through an increase in the recruitment of CBP/p300 protein to the androgen responsive elements in the promoters of androgen responsive genes. Down regulation of CBP/p300 expression using CBP/p300 specific siRNA abolished IL-4 mediated AR activation, suggesting that CBP/p300 is responsible for AR activation induced by IL-4. Furthermore, AR activation can be enhanced by AR acetylation induced by IL-4 in prostate cancer cells. The IL-4 mediated AR acetylation can be blocked by knocking down CBP/p300 expression using CBP/p300 specific siRNA.

CONCLUSION. These results suggest that IL-4 activates AR through enhanced expression of CBP/p300 and its histone acetyltransferase activity. *Prostate* 69: 126–132, 2009.

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KEY WORDS: interleukin-4; CBP/p300; AR; prostate cancer

INTRODUCTION

Interleukin-4 (IL-4) is a pleiotropic type I cytokine produced by T cells, mast cells, and basophils in response to receptor-mediated activation events [1]. IL-4 exerts biological effects on many immune cells, including B and T lymphocytes, mast cells, and macrophages, and plays a central role in regulating inflammatory and cell-mediated immune responses [2]. In addition to its effect on immune cells, IL-4 has a variety of other functions including effects on hematopoietic tissues, tissue adhesion and inflammation [2]. IL-4 functions through activation of the IL-4 receptor, designated IL-4R α , by tyrosine phosphorylation [3]. The receptor consists of a 140-kDa IL-4R α chain that binds IL-4 with high affinity (Kd 20–300 pM). IL-4R α

activation results in tyrosine phosphorylation of multiple receptor-associated kinases including Janus-family (Jak) tyrosine kinases (Jak1, Jak2, and Jak3) [4–6], insulin receptor substrate (IRS-1/2) proteins [7,8], Shc [9], and

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signal transducers and activators of transcription (Stat6) [10] for the initiation of signal transduction.

Prostate cancer cells depend on androgen for growth. Androgen regulates the expression of androgen-regulated genes such as prostate-specific antigen (PSA) through the binding of the androgen receptor (AR) to the androgen-responsive elements (AREs) in the promoter of the PSA gene. When androgen is depleted, prostate cancer cells initially undergo apoptosis and die. However, most patients will relapse with progression to ablation resistant disease due to the growth of castration recurrent cancer cells. Accumulating evidence demonstrates that abnormal AR signaling contributes to castration-resistant growth of prostate cancer. AR can be activated by growth factors and cytokines to display enhanced activity in the presence of low levels of androgen or to function even in the absence of androgen [11–16]. The formation of transcriptional complexes containing AR and coregulators such as CBP/p300 in the promoter and enhancer regions of AR responsive genes is required for maximum activation of androgen responsive genes. The coactivator proteins, cAMP-response element-binding protein (CBP) and the related functional homologue p300, regulate AR target gene expression. CBP/p300 was originally identified as a protein that bound to the adenoviral E1A and cAMP-response element-binding protein (CREB) [17,18]. CBP/p300 exhibits intrinsic and “associate” histone acetyltransferase (HAT) activity. CBP/p300 acetylates histones and facilitates binding of transcription factors to specific target DNA sequences by destabilizing nucleosomes bound to the promoter region of a target gene [19,20]. In addition, CBP/p300 was shown to acetylate non-histone proteins including nuclear receptors and transcription factors [21,22]. AR activity is increased by acetylation [23–26]. Recent evidence indicates that the expression of CBP/p300 genes is altered in various human tumors including prostate [27–30]. The correlation between p300 expression and higher Gleason scores in prostate cancer has also been reported [31,32].

Clinical data showed that the levels of IL-4 are significantly elevated in sera of patients with castration resistant prostate cancer [33,34]. Previous studies demonstrated that IL-4 activates AR-mediated gene expression by activation of the AR in a ligand-independent manner or in the presence of very low levels of androgen [35]. However, the mechanism involved in IL-4 mediated AR activation has not yet been revealed. In the present study, we investigated whether IL-4 induced AR activation in androgen depleted condition is through up regulation of CBP/p300 expression and increase of CBP/p300 HAT activity.

METHODS

Cell Culture

LNCaP cells were grown in RPMI 1640 with 10% FBS and penicillin/streptomycin at 37°C in a humidified atmosphere of 5% carbon dioxide. IL-4 over expressing LNCaP cells (C4A and C6A) were grown in RPMI 1640 media with 10% FBS containing G418 (300 µg/ml) [36].

Total and Nuclear Lysate Preparation

Whole cell extracts were obtained using High salt buffer with freeze-thaw procedure. For nuclear lysate preparation, cells were harvested, washed with PBS once and resuspended in a hypotonic buffer (10 mM HEPES-KOH (pH7.9), 10 mM KCl, 0.1 mM EDTA, 0.4% NP40, 0.5 mM PMSF, 0.5 mM DTT, 1 mM NaV, 20 mM NaF and 1 µg/ml protease inhibitor) and incubated on ice for 20 min. Nuclei were precipitated by centrifugation at 10,000 rpm at 4°C for 10 min. After washing once with hypotonic buffer, the nuclei were lysed in a high salt buffer (10 mM HEPES-KOH (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 mM NaV, 20 mM NaF, 20% glycerol and 1 µg/ml protease inhibitor) and incubated at 4°C for 30 min with vigorous shaking. The nuclear lysate was cleared by 12,000 rpm centrifugation at 4°C for 15 min. Protein concentration was determined using the Coomassie Plus protein assay kit (Pierce, Rockford, IL).

Electromobility Shift Assay

Electromobility shift assays were performed by incubating nuclear extracts (5–10 µg) with AR consensus binding motif 5'-GGTACAGGGTGTCT-3' (Santa Cruz Biotechnology, Santa Cruz, CA) in incubation buffer containing 10 mM HEPES pH 7.9, 80 mM NaCl, 10% glycerol, 1 mM DTT, 1 mM EDTA, 100 µg/ml poly(dI-dC). The protein–DNA complexes were resolved on a 4.5% non-denaturing polyacrylamide gel at room temperature, and the results were autoradiographed using Storm 840 Imaging system (Molecular Dynamics, Sunnyvale, CA).

Transfection and Reporter Gene Assay

1×10^5 cells (LNCaP and IL-4 over expressing cells) were plated in 6-well plate. After 24 hr, cells were transiently transfected with ARE-luc using Tfx 20 (Promega, Madison, WI) according to the manufacturer's instructions. If necessary, AR was cotransfected with ARE-luc. After 1 hr of transfection, the DNA: liposome mixture was removed, and the cells were treated with phenol red-free medium containing 5% CS-FBS with 0, 0.1, 1, and 10 nM of R1881. Cell

extracts were obtained 36 hr later and luciferase activity was assayed using the Luciferase Assay System (Promega). All transfection experiments were performed in triplicate wells and repeated at least four times. For CBP/p300 siRNA transfection, cells (2×10^6) were plated in 100 mm culture dish and transfected with either CBP/p300siRNA or control siRNA. Cells were harvested and subjected to total cellular and nuclear protein extraction after incubating overnight either in regular FBS medium or medium containing charcoal-stripped FBS.

Western Blot analysis

Whole cell extracts or nuclear extracts were resolved on 10% or 12% SDS-PAGE. Proteins were then transferred to nitrocellulose membrane. After blocking overnight at 4°C in 5% milk in PBS-0.1% Tween20, membranes were incubated for 1 hr at room temperature with antibodies against AR or CBP/p300 (Santa Cruz Biotechnology) in 1% milk in PBS-Tween 20. Following incubation with secondary antibody, immunoreactive proteins were visualized with an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Buckinghamshire, England).

Chromatin Immunoprecipitation (ChIP) Assay

Protein-DNA complexes in LNCaP, neo, IL-4 over expressing cells were cross-linked inside the cells by the addition of formaldehyde (1% final concentration) to the cells in culture. Whole cell extracts were prepared using sonication and an aliquot of the cross-linked receptor DNA complexes were immunoprecipitated by incubating with CBP/p300 specific antibodies overnight at 4°C with rotation. Chromatin-antibody complexes were isolated from solution by incubation with protein A/G-Sepharose beads for 1 hr at 4°C with rotation. The Sepharose bound immune complexes were washed and eluted from beads with elution buffer (1% SDS and 0.1 mM NaHCO₃), and DNA was extracted. DNA samples from chromatin immunoprecipitation preparations were analyzed by PCR using primers spanning androgen-responsive elements in the promoter of the PSA gene (forward, 5'-CCTAGATGAAGTCTCCATGAGCTACA; reverse, 5'-GGGAGGGAGAGCTAGCACTTG) as described previously [37].

Immunoprecipitation Study

Whole cell extracts were prepared from neo and IL-4 over expressing LNCaP cells that were grown either in normal medium or in medium containing charcoal-stripped FBS. Immunoprecipitation was carried out either with acetylated lysine antibodies (Upstate Bio-

technology, Lake Placid, NY) or with CBP/p300. The immunocomplexes were separated by SDS-PAGE and analyzed using antibodies against AR.

RESULTS

IL-4 Enhances CBP/p300 Expression

We have previously demonstrated that IL-4 activates AR signaling and facilitates survival of LNCaP cells in androgen depleted environment in vitro and in vivo [35,36]. In order to elucidate the mechanism of IL-4 mediated AR activation, we examined the effects of IL-4 on the expression of several coactivators including CBP/p300. Nuclear extracts were obtained from IL-4 treated LNCaP cells that were grown in androgen depleted charcoal stripped FBS medium and the levels of CBP/p300 protein were examined by Western blot analysis. IL-4 enhanced the levels of CBP/p300 protein expression in a dose-dependent manner (Fig. 1A). We also examined CBP/p300 expression in IL-4 overexpressing LNCaP clones. The development and characteristics of C4A and C6A were described previously [36]. Similarly, the levels of CBP/p300 protein expression were considerably elevated in IL-4

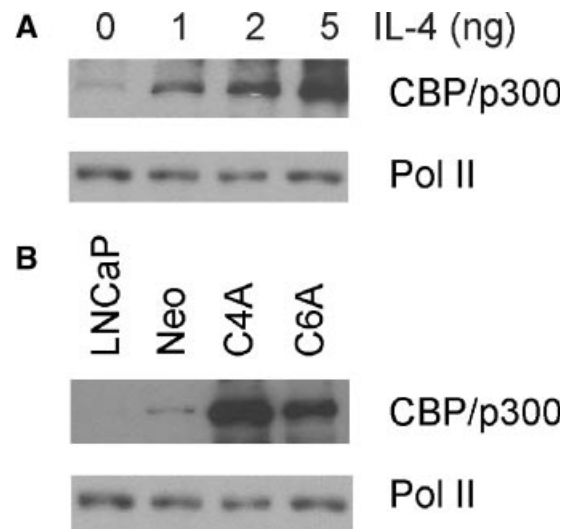


Fig. 1. IL-4 increases CBP/p300 expression. **A:** LNCaP cells (2×10^6) were plated in 100 mm culture dishes. Twenty-four hours before treatment with IL-4, the medium was changed into the one containing charcoal stripped-FBS. IL-4 (0, 1, 2, 5 ng/ml) was added into the medium and incubated for 24 hr. Cells were then harvested and nuclear extracts were obtained. The expression of CBP/p300 in nuclear fraction was analyzed by Western blotting using antibodies against CBP/p300. **B:** LNCaP, neo and IL-4 over expressing LNCaP cells (C4A, C6A) were grown in androgen depleted (charcoal stripped FBS containing) medium for 2 days. Nuclear proteins were harvested and the expression of CBP/p300 was analyzed by Western blot analysis using antibodies against CBP/p300. Pol II was used for nuclear protein loading control.

over expressing LNCaP clones C4A and C6A compared to the parental LNCaP cells and vector control clones (Fig. 1B). Collectively, these results suggest that overexpression of IL-4 enhances CBP/p300 protein expression in LNCaP cells.

Activation of AR by IL-4 Is Mediated by CBP/p300

CBP/p300 is a coactivator that can enhance AR activation. Since IL-4 activates AR signaling and enhances CBP/p300 expression, we next examined whether CBP/p300 is responsible for IL-4 mediated AR activation. We introduced CBP/p300 specific siRNA into IL-4 overexpressing cells to examine whether the activation of AR by IL-4 in androgen depleted conditions can be inhibited by downregulation of CBP/p300 expression. The transcriptional activity of ARE-luc was considerably decreased when the IL-4 overexpressing cells were transfected with CBP/p300 specific siRNA in androgen depleted conditions (Fig. 2A). The binding of AR to ARE DNA motif in cells overexpressing IL-4 was also decreased by knocking down CBP/p300 expression using its specific siRNA (Fig. 2B). These results suggest that IL-4 mediated AR activation in androgen depleted condition is mediated at least in part through enhanced expression of CBP/p300 in LNCaP cells.

IL-4 Enhances the Recruitment of CBP/p300 Protein to the AR/ARE Complexes

Having demonstrated that IL-4 enhances CBP/p300 protein expression and involvement of CBP/p300 in IL-4 mediated AR activation, we next examined whether IL-4 enhances the interaction between AR and CBP/p300 proteins by co-immunoprecipitation assays. As shown in Figure 3A, AR:CBP/p300 protein complexes were formed in both LNCaP parental cells and IL-4 over expressing LNCaP cells. However, intensity of the complexes in IL-4 over expressing LNCaP cells was much stronger compared to the parental LNCaP cells.

To determine whether IL-4 enhances the recruitment of CBP/p300 to AR/ARE complexes, chromatin immunoprecipitation (ChIP) assays were performed. The cell extracts containing cross-linked protein-DNA complexes were immunoprecipitated with anti-CBP/p300 antibodies and the resultant chromatin was amplified using primers spanning the AREs in the promoter of PSA gene. As shown in Figure 3B, IL-4 increased the recruitment of CBP/p300 to the ARE promoter regions. Antibodies against CBP/p300 precipitated fragments containing the ARE regions in both IL-4 overexpressing LNCaP cells and parental LNCaP cells. However, the recruitment of CBP/p300 to the AREs was much higher in IL-4 overexpressing

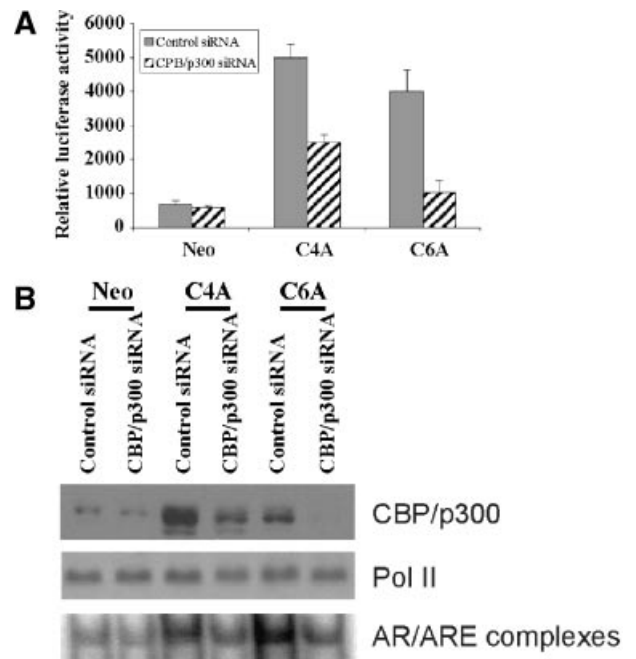


Fig. 2. Knockdown of CBP/p300 expression inhibited IL-4 mediated AR activity. **A:** Knockdown of CBP/p300 expression inhibited IL-4 mediated ARE activity. Neo and IL-4 over expressing LNCaP cells (C4A and C6A) were plated in 6-well culture plates and cotransfected with ARE-luc and 0.5 μ g of CBP/p300 siRNA or with equal amount of control siRNA [30]. After transfection, cells were incubated in charcoal stripped FBS containing medium with 0.1 nM of R1881. Forty hours later, cells were harvested and luciferase activity was measured. Data were derived from a representative experiment performed in triplicate after normalization to *Renilla* luciferase activity (internal control). Each bar is mean \pm SD. **B:** Effect of knockdown of CBP/p300 expression on AR/ARE binding activity by electromobility shift assay (EMSA). Neo and IL-4 over expressing LNCaP cells (C4A and C6A) were plated in 100 mm culture dishes and transfected with either 5 μ g CBP/p300 siRNA or equal amount of control siRNA as described above. Twenty-four hours later, cells were harvested and nuclear extracts were obtained. The nuclear extracts were used for Western blot analysis using antibodies against CBP/p300 and EMSA analysis using ARE DNA binding motif as a probe as described previously [35].

LNCaP cells compared to the parental LNCaP cells (Fig. 3B). Taken together, these results suggest that IL-4 mediated AR activation is through enhanced expression and recruitment of CBP/p300 to the AR/ARE complexes.

IL-4 Induces AR Acetylation Mediated by CBP/p300

CBP/p300 has intrinsic HAT activity and is known to acetylate nuclear receptors [19,24]. To examine whether acetylation enhances AR activation, LNCaP cells were cultured in media containing 10% charcoal stripped FBS in the presence of 0.01 nM R1881 and transfected with ARE-Luc reporter and treated with

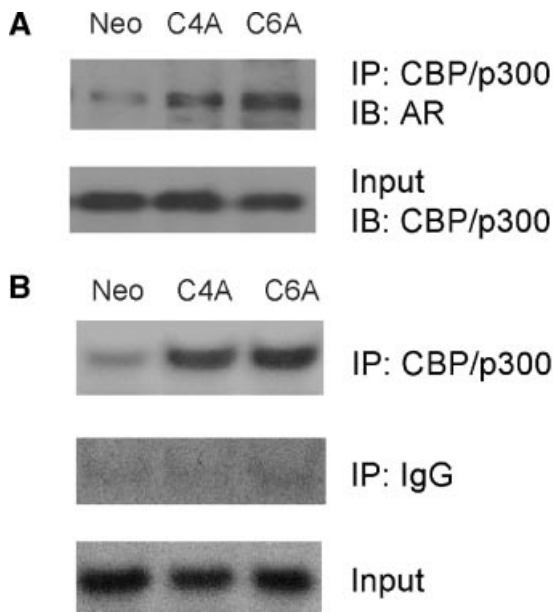


Fig. 3. IL-4 enhances the interaction between AR and CBP/p300 protein. **A:** Overexpression of IL-4 increases the interaction of AR with CBP/p300. Whole cell extracts were prepared from neo and IL-4 over expressing (C4A and C6A) LNCaP cells. Immunoprecipitation was carried out with antibodies against CBP/p300. The immunocomplexes were separated by SDS-PAGE and analyzed using antibodies against AR. **B:** Overexpression of IL-4 increases the recruitment of CBP/p300 to ARE site. IL-4 over expressing cells (C4A and C6A) and neo control were crosslinked using formaldehyde. After harvesting cells, sonication was performed and 10 μ g of proteins were used for immunoprecipitation using antibodies against either CBP/p300 or IgG as a control. After reversing cross-linking, bound DNA was extracted and used for PCR analysis using primers spanning AREs in the promoter region of PSA gene as described previously [37].

increasing doses of trichostatin A (TSA), a broad inhibitor of histone deacetylases. TSA treatment increased AR expression and induced ARE luciferase activity in a dose-dependent manner (Fig. 4A). Since IL-4 enhances CBP/p300 protein expression, we examined whether AR acetylation is increased by IL-4 using immunoprecipitation assay. LNCaP parental and its IL-4 over expressing cells were grown in androgen deprived conditions and immunoprecipitated with antibodies against acetylated lysine. The immune complexes obtained were analyzed by Western blotting using antibodies against AR. Figure 4B shows enhanced acetylation of AR in IL-4 over expressing cells, C4A and C6A, compared to the parental LNCaP cells. We next examined whether IL-4 induced AR acetylation is mediated by elevated expression of CBP/p300 protein. IL-4 overexpressing LNCaP cells were transfected with CBP/p300 specific siRNA or control scramble siRNA and the cell lysates were analyzed by immunoprecipitation. Down-

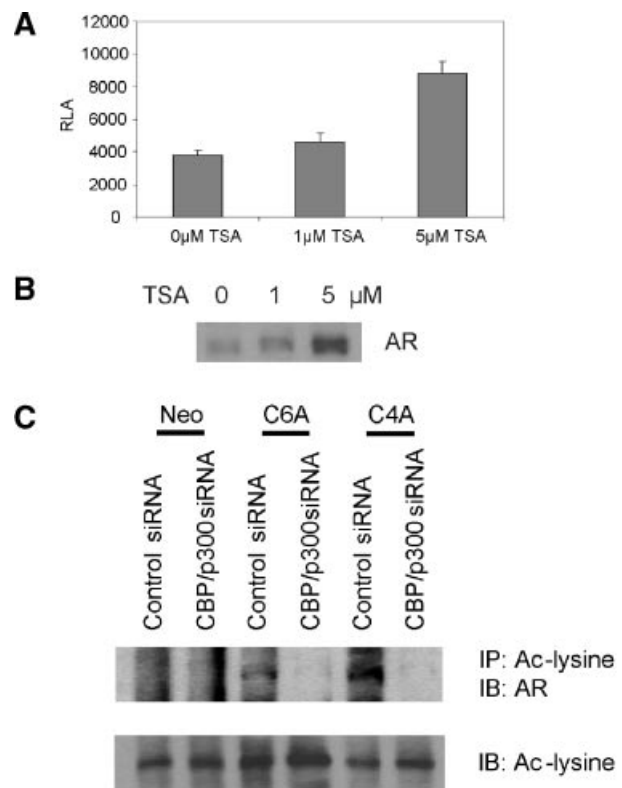


Fig. 4. CBP/p300 mediates IL-4 induced AR acetylation. **A:** TSA treatment increases ARE activity. LNCaP cells were cultured in androgen deprived conditions in the presence of 0.01 nM R1881 and transfected with ARE-luc reporter. The cells were treated with increasing doses of TSA as indicated and luciferase activity was measured. Data were derived from a representative experiment in triplicate after normalization to *Renilla* luciferase activity (internal control). Each bar is mean \pm SD. **B:** IL-4 increases AR acetylation. Total lysates (100 μ g) obtained either from neo or IL-4 over expressing C4A and C6A cells were precleared and immunoprecipitated with antibodies against acetylated Lys residue. Proteins released from precipitated ProteinA-agarose bound immunoprecipitates were separated by SDS-PAGE and Western blot was performed using antibodies against AR. **C:** Cells (neo and IL-4 over expressing C4A and C6A cells) were transfected with either CBP/p300 siRNA or control siRNA. Twenty-four hours later, total lysates were obtained and were subjected to immunoprecipitation as described above.

regulation of CBP/p300 expression by CBP/p300 specific siRNA significantly decreased the levels of AR acetylation induced by IL-4 compared to the control scramble siRNA (Fig. 4C). These results suggest that AR undergoes CBP/p300-mediated acetylation induced by IL-4.

DISCUSSION

Clinical investigations showed that the levels of IL-4 are significantly elevated in sera of patients with hormone refractory prostate cancer compared to levels

in hormone-sensitive prostate cancer, and that the levels of IL-4 were directly correlated with elevated PSA [33,34]. Previous studies demonstrated that IL-4 enhances PSA expression by activation of AR mediated in part by the activation of NF- κ B [35,38]. Further studies showed that IL-4 plays a critical role in promoting castration-resistant growth of androgen-responsive LNCaP prostate cancer cells [36]. In the present study, we investigated the role of CBP/p300 in IL-4 mediated AR activation. We demonstrated that IL-4 enhances CBP/p300 protein expression and suggested a critical role of CBP/p300 in IL-4 induced AR activation by increasing AR acetylation.

The present study demonstrated that IL-4 enhances CBP/p300 protein expression in prostate cancer cells. The induction of CBP/p300 expression by IL-4 was observed in androgen deprived conditions when LNCaP prostate cancer cells were treated with exogenous IL-4 and also when IL-4 was ectopically expressed in LNCaP cells by transfection with IL-4 cDNA. Furthermore, IL-4 enhanced the interactions between CBP/p300 and AR proteins as shown by co-IP assays, and increased the recruitment of CBP/p300 protein to the ARE sites in the promoter of androgen responsive genes by ChIP assays. These results suggest that IL-4 induction of AR activation may be through enhancement of CBP/p300 expression and subsequent recruitment of CBP/p300 to the ARE sites. This was confirmed in LNCaP cells in which IL-4 induced AR activation was abolished by knocking down CBP/p300 expression using siRNA.

The coactivator CBP/p300 interacts with the AR and enhances androgen-mediated AR activation. CBP/p300 has intrinsic and "associated" histone acetyltransferase (HAT) activity that regulates gene expression by acetylating histones, which facilitates the displacement of nucleosomes on the DNA and favors the binding of transcription factors to specific target DNA sequences. Accumulating evidence demonstrates that CBP/p300 plays an important role in the androgen-dependent activation of the AR through its intrinsic and "associated" HAT activity to acetylate the AR at sites governing hormone-dependent transactivation [26]. It is also demonstrated that CBP/p300 mediates AR activation by IL-6 in a ligand-independent manner in prostate cancer cells [23]. In the present study, we demonstrated that IL-4 enhances AR acetylation that is mediated by CBP/p300 HAT activity. Knock down of CBP/p300 expression by siRNA blocked AR acetylation and ligand-independent transactivation induced by IL-4 in prostate cancer cells.

Both ligand-dependent and -independent mechanisms are involved in the activation of AR. Recent studies showed that IL-6 induced ligand-independent

AR activation and PSA expression were mediated by CBP/p300 HAT activity [39]. The fact that IL-4 induced ligand-independent AR activation is also through an increase in the CBP/p300 HAT activity suggests that alteration of CBP/p300 HAT activity is a general mechanism associated with ligand-independent AR activation, and that it may play a critical role in the eventual progression to a castration-resistant state.

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