Inhibition of MAPK-signaling pathway promotes the interaction of the corepressor SMRT with the human androgen receptor and mediates repression of prostate cancer cell growth in the presence of antiandrogens

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Abstract

Prostate cancer is one of the most prominent malignancies of elderly males. The growth of normal prostate and prostate cancer (PCa) cells depend on functional androgen receptor (AR), a ligand controlled transcription factor and member of the nuclear receptor superfamily. Binding of agonistic ligand enhances the transactivation function of AR and hence promotes the growth of prostate epithelial cells. We have earlier shown that AR antagonistic ligands such as cyproterone acetate (CPA) promote the recruitment of transcriptional corepressors such as silencing mediator of retinoid and thyroid receptor (SMRT) leading to repression of AR transactivation in non-PCa cells. Unfortunately, however, in LNCaP PCa cells, CPA functions as an agonist and thereby increases AR transactivation function. Here, we show that activated MEK signaling cascade inhibits functional recruitment of corepressor SMRT to CPA-bound AR in PCa cells. Chemical blockade of MEK kinase using a specific inhibitor U0126 increases the interaction and hence repression of AR by the corepressor SMRT in LNCaP PCa cells. This inhibition also results in enhanced antagonistic behavior of CPA as assessed by reporter and cell-growth assays. Moreover, the growth of LNCaP cells stably overexpressing SMRT was more robustly inhibited in the presence of CPA and U1026. In line with this, the growth rate of LNCaP cells was decelerated in the presence of both CPA and U0126. This suggests that activated MEK signaling pathway attenuates the functional recruitment of corepressor SMRT to AR induced by antagonists and thus indicates the important role of corepressors in mediating repression of both AR transactivation and PCa cell growth by antagonists. Furthermore, these findings suggest that combining receptor antagonists with signaling inhibitors could be a beneficial approach for PCa treatment.

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Introduction

Prostate cancer (PCa) is the most often diagnosed cancer in males and the second cause of male cancer death in western countries (Jemal et al. 2008). The growth of the normal prostate as well as of PCa is regulated by androgens acting through androgen receptor (AR). Activated AR promotes PCa cell growth and thus AR represents a major drug target in the treatment of PCa (Dehm & Tindall 2007). AR binds to androgen response elements (ARE) and activates its target genes, such as the PSA used as a diagnostic marker for PCa.

Inhibition of AR-activity by reducing endogenous androgen production and treatment of patients with AR antagonists (antihormones) is a major goal in the hormone therapy. Antihormones mediate their inhibitory function on AR in part through recruitment of corepressors to the AR, for example, the clinically used cyproterone acetate (CPA; Dotzlaw et al. 2002, 2003, Moehren et al. 2007). CPA acts as a partial agonist for the wild-type AR leading to AR-mediated transactivation, which is weaker compared with that induced by pure agonists. Corepressors are coregulators that themselves do not bind to DNA directly but are recruited by transcription factors and mediate gene repression (Burke & Baniahmad 2000, Heemers & Tindall 2007) by recruiting gene silencing machinery. A typical nuclear hormone receptor corepressor possesses nuclear receptor interaction domain(s) and silencing domain(s). It could be shown that the inhibitory activity of CPA is dependent on recruitment of corepressors, such as silencing
mediator of retinoid and thyroid receptor (SMRT), to AR and that the level of agonistic activity of CPA can be dictated by the corepressor availability (Dotzlaw et al. 2002, Moehren et al. 2004).

However, after a short period of initial responsiveness to antiandrogen therapy, eventually the emergence of androgen-independent tumor follows leading to androgen refractory disease. Several mechanisms have been shown to be involved in this transition such as increased AR protein levels in PCa cells, mutations of AR, activated signaling pathways, and altering the ratio of coregulators towards an overabundance of coactivators (Chen et al. 2004, Dehm & Tindall 2007). Furthermore, reduced recruitment of corepressors seems also to play a likewise important role and accounts for elevated AR activity despite the presence of AR antagonists (Dotzlaw et al. 2002, Dehm & Tindall 2007). One potential mechanism leading to decreased corepressor recruitment was discussed to be the aberrant activation of signaling pathways (Graham et al. 2000, Dotzlaw et al. 2002, Asim et al. 2008).

In previous experiments, we found that the corepressor SMRT binds to AR in the non-PCa CV1 cells and thus potentially serves as a model for corepressor binding to AR and for mediating anti-hormone responsiveness (Dotzlaw et al. 2002, 2003). However, we did not observe recruitment of ectopically expressed or endogenous SMRT to AR in LNCaP cells that serve as a model for androgen-dependent human PCa. As survival and growth of cells are generally highly dependent on mitogens and the stimulation of the MAPK signaling pathway and an activation of this signaling cascade has been associated with PCa progression (Gioeli et al. 1999), we hypothesized that an aberrantly activated MAPK pathway in LNCaP might prevent SMRT recruitment to antagonist-bound AR and be an underlying mechanism of acquired drug resistance in PCa.

Here, we show that the MEK inhibitor U0126 induces the interaction of SMRT with AR in LNCaP cells in the presence of CPA suggesting that the MAPK signaling reduces SMRT recruitment to CPA-bound AR. Co-treatment of LNCaP cells with CPA and U0126 results also in further lowering the endogenous PSA expression and cell growth. This suggests that combining AR antagonists with signaling inhibitors could enhance anti-hormone action in PCa by functionally activating corepressor function.

Material and methods

Cell culture, transient and stable transfection, growth analyses

CV1 and LNCaP cells were grown as previously described (Moehren et al. 2007). Transfections were performed using a modified calcium-phosphate method (Dotzlaw et al. 2003). The mammalian one-hybrid system and the VP16 and VP16-SMRT vectors were described earlier (Dotzlaw et al. 2002, Moehren et al. 2008). U0126 and CPA were obtained from Sigma. For reporter assays, cells were harvested 72 h post-transfection and both luciferase and β-galactosidase activity were measured. Independent triplicate experiments were performed each time and were repeated at least three times. Student’s t-test was used for statistics indicated by P-values of normalized units.

For cell number detection, triplicates were untreated or treated in 24-well and 6-well plates and counted at indicated days. The experiments were repeated three times. The mean and the deviation of the mean are depicted.

For analysis of AR protein levels, LNCaP cells were treated with U0126 for 72 h prior to harvest and western analysis of whole cell extracts. Antibodies used were mouse anti-AR (Biogenex Laboratories Inc., San Ramon, CA, USA), anti-β-actin (13E5) rabbit (Cell Signaling Biotechnology, Danvers, MA, USA), anti-mouse IgG, HRP from goat and anti-rabbit IgG HRP from bovine were used as secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Normalizations were calculated via ImageJ Software (National Institutes of Health, Scion Corporation, Maryland, USA).

Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) was performed according to Moehren et al. 2007 using LNCaP cells treated with CPA (10⁻⁷ M) or with CPA (10⁻⁷ M) and U0126 (10⁻⁵ M) solved in ethanol, and harvested 30 min after application using anti-AR, PG-21, polyclonal, (Upstate, Temecula, CA, USA). Washing buffers contained CPA (10⁻⁷ M) or CPA (10⁻⁷ M) and U0126 (10⁻⁵ M). Co-immunoprecipitated proteins were transferred on nitrocellulose membrane (Millipore). Anti-SMRT polyclonal (Affinity BioReagents, Rockford, IL, USA); anti-rabbit IgG, HRP from bovine was purchased from Santa Cruz Biotechnology.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) experiment was performed essentially as described previously (Moehren et al. 2007). Anti-AR antibody (PG-21; Upstate) anti-SMRT (PA1-842; Affinity BioReagents) and IgG (Santa Cruz Biotechnology). The following primer pair was used to test the recruitment of AR and corepressor SMRT on the enhancer of the PSA gene: PSA ARE III Fwd: 5’ ATC CTC AAT CTT ATA CTG GGA C 3’; PSA ARE III Rev: 5’AAG GCT CTG GCTG AAC AGT G 3’.
Real time RT-PCR

Isolation of mRNA and the real-time PCR was performed as described earlier (Moehren et al. 2007). LNCaP cells were treated as indicated for 3 days, total cellular RNA was isolated, 1 μg RNA was reverse-transcribed to cDNA and was subjected to amplification by light cycler (Roche) using specific primers and control primers against PSA and β-actin.

Generation of stable clones

LNCaP cells were transfected with pCMX-SMRT full-length along with pETE-Hyg plasmid in 1:1 molar ratio with DOTAP transfection method according to manufacturer’s protocol (Carl Roth GmbH & Co., Karlsruhe, Germany). Cells were selected with hygromycin (0.2 mg/ml; Sigma). Untransfected cells served as control. Number of colonies was counted after 2–3 weeks of incubation.

Results

The MEK inhibitor U0126 restores interaction of the corepressor SMRT with AR

To test for the influence of the MEK inhibitor on the interaction of SMRT with the AR, the mammalian one-hybrid assay (Dotzlaw et al. 2002, 2003, Moehren et al. 2007) was performed. LNCaP cells were transfected with the androgen responsive MMTV-reporter together with either expression vector coding for the transactivation domain VP16 alone or the VP16-cSMRT fusion, lacking the SMRT silencing domains but retaining the receptor interaction domain. An interaction between the endogenously expressed AR and SMRT resulted in a ligand-dependent increase of reporter activity. Cells were treated with or without the AR antagonist CPA and/or U1026, a specific MEK1/2/5 inhibitor (Nishimoto & Nishida 2006). In the absence of U0126, only weak interaction was observed between SMRT and AR whereas treating cells with U0126 resulted in enhanced interaction as reflected in an increase (P>0.02) of the reporter activity (Fig. 1) suggesting that activating MEK kinase in LNCaP cells opposes interaction of corepressor SMRT with AR. On the other hand, the tyrosine kinase inhibitor, AG1517, did not restore the SMRT-AR interaction in these assays, indicating a specific functional role of MAPK signaling in regulating corepressor action on AR.

To verify an increase in interaction between SMRT and AR on the endogenous PSA gene, ChIP experiments were used and the recruitment of AR and SMRT was analyzed on the PSA enhancer in LNCaP cells treated with or without CPA or U1026 in vivo (Fig. 2A). The recruitment of AR was increased in the presence of CPA, which is in line with previous data (Moehren et al. 2007). The interaction of AR remained unchanged employing U0126. By contrast, only a weak recruitment was observed for SMRT in the presence of CPA alone, which was enhanced by additional treatment of cells with U0126. These findings suggest that the recruitment of SMRT is regulated both by the AR antagonist and MEK-signaling pathway in PCa cells.

Furthermore, co-immunoprecipitation experiments (Co-IP) using LNCaP cells with or without U0126 in the presence of CPA suggest an enhanced interaction of endogenous SMRT with the endogenous hAR in the presence of U0126 (Fig. 2B).

Thus, these findings support the notion that activated MEK-signaling inhibits the interaction and recruitment of SMRT to the hAR.

Combined treatment of MEK inhibitor U0126 and the AR antagonist CPA inhibits AR-mediated transactivation

To test whether the increase in interaction between SMRT and AR results in the repression of AR-mediated transactivation. LNCaP cells were cotransfected with the MMTV-luc reporter and the expression vector for SMRT and empty vector as control. Cells were treated in combination with or without CPA and U0126. The transfection with the SMRT expression vector slightly
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Figure 2 SMRT interaction and recruitment is enhanced by U0126 in the presence of the AR ligand CPA on the PSA enhancer. (A) Chromatin immunoprecipitation (ChIP) was performed using LNCaP cells treated with CPA (10^{-7} M) and/or U0126 (10^{-7} M) for 30 min using antibodies against SMRT, AR or IgG as control. PCR products were analyzed by gel electrophoresis and visualized with a fluorescence sensitive gel scanner. Presence of PCR product band reflects the recruitment of investigated protein to the PSA enhancer region. (B) Co-immunoprecipitation (Co-IP) was performed with LNCaP cells using anti-AR antibody (IP) and detection of SMRT by western blotting (WB) in the presence of CPA (10^{-7} M) and/or U0126 (10^{-7} M).

Figure 3 Treatment with U0126 increases SMRT-mediated repression of CPA-activated AR. LNCaP cells were transfected with 2 µg expression vector encoding full-length SMRT or corresponding empty vector along with 1 µg reporter construct pMMTV-Luc and 0.2 µg internal control vector pCMV-LacZ. Cells were incubated for 72 h in the presence or absence of the partial antagonist CPA (10^{-7} M) and U0126 (10^{-5} M). The obtained luciferase values (RLU) were normalized to the corresponding β-galactosidase. The value obtained in the absence of hormone and inhibitor was arbitrarily set as one and used as reference for all other values.

Stable integration of SMRT inhibits potently LNCaP colony formation in the presence of both U0126 and CPA

To test whether the inhibition of AR-mediated transactivation is associated with changes in cell growth, LNCaP cells were treated with CPA and/or U0126 individually or combined and total cell numbers were determined and plotted as relative cell number (Fig. 5A). To ensure survival of androgen-dependent LNCaP cells were grown in medium containing normal untreated serum, known to contain androgens (Moehren et al. 2007). Compared with control, the treatment with CPA did not change the growth rate of LNCaP cells. Expectedly, treatment with the mitogen signaling cascade inhibitor U0126 reduced the growth of LNCaP cells (P<0.002), which was significantly further reduced by co-treatment with U0126 with CPA (P<0.01; Fig. 5A). These data suggest that the combined treatment of the signaling inhibitor U0126 acts in concert with the AR antagonist CPA to reduce LNCaP PCa-cell proliferation at least in part by activation of corepressor SMRT action on CPA-bound AR.

To reveal the functional role of the corepressor SMRT on the CPA and U0126 induced growth inhibition, we stably transfected LNCaP cells with either

reduced the reporter activity in the presence of CPA and more potently when cells were co-treated with CPA and U0126 (P<0.01 comparing empty vector control with SMRT expression vector) (Fig. 3). Since the basal level activities without CPA treatment were not significantly affected, it suggests that the observed effects are CPA-dependent. These data indicate that the AR-mediated transactivation is repressed through the corepressor SMRT, the antagonist CPA and by the MAPK-signaling inhibitor U0126.

To verify the effects on the expression of the endogenous PSA gene, LNCaP cells were treated with U0126 alone or in combination with CPA. Real-time RT-PCR experiments were performed to detect the expression of PSA mRNA (Fig. 4A). As expected, the treatment with the antagonist CPA, which is known to have partial agonistic activity, increased PSA expression levels (P>0.005). Treatment with U0126 itself had no significant effect on PSA mRNA levels, whereas the combined treatment with CPA and U0126 reduced the mRNA levels (P>0.01). Furthermore, we tested whether U0126 might affect the AR protein stability. LNCaP cells were treated with U0126 for 72 h and the endogenous AR levels were detected by western analyses with anti-AR antibody (Fig. 4B). The data suggest that U0126 treatment does not influence AR protein level. These findings suggest that co-treatment with CPA and U0126 synergistically repress PSA expression and indicate that AR-mediated transactivation can be efficiently repressed by the combination of the AR antagonist and signaling inhibitor U0126.
empty control or SMRT expression vector or allowed cell growth in the presence of the AR antagonist and/or MEK inhibitor. Cells were continuously selected, sustained, and treated again in low percentage normal untreated serum containing medium. The formed individual cell colonies provide a measure for cell survival and their growth potential. Transfection of the SMRT expression vector per se resulted in a dramatically decreased number of cell colonies when compared with empty vector control ($P > 0.001$), suggesting that the expression of the corepressor efficiently inhibits growth-promoting factors (Fig. 5B). This can be explained by the fact that SMRT is not an AR specific corepressor but also interacts with several other cell cycle regulators (Feng et al. 2001, Li & McDonnell 2002). Treatment with U0126 alone only slightly reduced the colony number, whereas cells that were stably transfected with SMRT exhibited a more reduced colony formation potential ($P > 0.02$). Most notably, treatment with CPA in the presence of U0126 further reduced colony number ($P > 0.02$). These findings suggest that although SMRT overexpression and its subsequent activation by MEK inhibition cooperates in repressing PCa cell growth by decreasing the transactivation of CPA-bound AR.

Taken together, our data suggest that activated signaling pathways, such as the MAPK signaling pathway, can reduce or abrogate the action of AR antagonists used in PCa antihormone therapy by functional inactivation of corepressors such as SMRT. Furthermore, our findings indicate that a combined treatment with the MAPK signaling inhibitor U0126 and the AR antagonist CPA can restore its growth inhibition by restoration of functional AR repression via the corepressor SMRT.

**Discussion**

This study investigated the role of MEK signaling in undermining antagonistic action of CPA against human PCa cells. We show that the recruitment of SMRT to the AR and hence the SMRT-mediated repression of AR transactivation in PCa cells is subjected to regulation by activated MEK signaling. The inhibitor U0126 was used to inhibit MEK activity. Although U0126 is described and widely used as a highly specific inhibitor of MEK1/2/5, with a high selectivity over other signaling kinases (Favata et al. 1998, Nishimoto & Nishida 2006) we do not rule out that other kinases might be affected.

Since the corepressor SMRT is recruited to and inhibits AR transactivation function by CPA in CV1 cells, it is thought that a decrease in SMRT recruitment could account for the weak inhibition of the AR by CPA in LNCaP cells. However, the endogenous mutant of AR in LNCaP cells (single amino acid exchange at T877A) is also efficiently binding to SMRT in the presence of CPA suggesting that the T877A mutation itself is not the cause for the deficiency in SMRT recruitment, since SMRT-AR T877A interaction is evident in CV1 cells (unpublished observations).

We observed a weak interaction of SMRT to AR in the presence of CPA, which was confirmed by ChIP, where
This suggests that MAPK activates AR-mediated transactivation. Moreover, stable integration of SMRT expression and treatment of PCa cells with CPA and U0126 further supports the notion that anti-androgen inhibition of proliferation is at least in part mediated by SMRT. This is in line with enhanced interaction of SMRT with AR. This does not rule out other pathways regulated by the MAPK such as corepressors or coregulators of AR. A potential mechanism of how SMRT is inactivated by the MEK signaling is that SMRT becomes phosphorylated in response to activated MEK signaling which is associated with export from the nucleus (Hong & Privalsky 2000). This pathway seems to prevent SMRT from interacting with the AR in LNCaP cells. Our results confirm this hypothesis that MEK activity is involved in the regulation of SMRT-AR interaction, since treatment with the inhibitor U0126 successfully restored the interaction in the presence of CPA. Notably, regaining the functional interaction of SMRT to AR is beneficial in such a way that AR is re-repressed in the presence of CPA and that this results in inhibition of PCa cell growth. Thus, linking inhibition of signaling pathway and regaining AR antagonism by CPA could serve as an important strategy to circumvent failure of CPA based antihormones therapy. In fact, it is possible that other signal transduction pathways could potentially be involved in regulating corepressor action on CPA bound AR. We have recently shown that blocking Src kinase activity in PCa cells inhibit AR transactivation function (Asim et al. 2008) presumably by activating endogenous corepressors to inhibit ligand bound AR function.

Recent studies support our findings that the activity of the AR in the presence of agonists is further enhanced by the activated Ras-MEK-MAPK pathway in PCa cells (Mukhopadhyay et al. 2007, Agoulnik et al. 2004, Lange et al. 2007). On the other hand, the inactivation of AR in the presence of AR antagonists remains a major problem in PCa therapy. Activation of AR in the presence of antagonists may counteract the success of antagonist treatment. Our data have the notion that one underlying molecular mechanism regulating the AR-mediated transactivation and androgen-regulated proliferation of LNCaP cells in the presence of antagonist is the reduced interaction of the corepressor SMRT with the human AR by the activated MAPK pathway.

Moreover, it has been shown that DHT can activate MEK signaling through non-genomic actions of the AR (Peterziel et al. 1999, Castoria et al. 2004, Lange et al. 2007).
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In combination with the data presented here, this would propose a mechanism in which DHT can actively influence the availability of SMRT for recruitment to the nuclear AR. Also, in light of this view inhibition of the MEK pathway enhances the corepressor function and AR antagonisms by antihormones.

In conclusion, this work reveals an important role for SMRT in the inhibition of the AR by CPA and provides explanation for the attenuated therapeutic benefits observed in a subset of PCa patients treated with CPA. Our data suggest a possible growth inhibitory effect of CPA in LNCaP cells is prevented by MEK signaling and indicates a possible improvement of PCa treatment by simultaneous inhibition of MEK signaling.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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