

Dihydrotestosterone interacts with EGFR/MAPK signalling and modulates EGFR levels in androgen receptor-positive LNCaP prostate cancer cells

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Abstract. Androgen receptor (AR) signalling plays a pivotal role in prostate cancer pathogenesis and progression. However, androgen-mediated AR signalling is yet to be fully understood. EGFR and MAP kinase signalling pathways play predominant roles in AR function. Therefore, we investigated the interaction of EGFR signalling and AR activity in AR-positive LNCaP cells. We found that 5 α -dihydrotestosterone (DHT) and EGF had a synergistic effect on AR activity as detected by a luciferase reporter system, although EGF alone did not activate AR. Both ERK1/2 and p38 were involved in DHT and DHT/EGF-induced AR activation as detected by specific MEK and p38 inhibitors. Furthermore, 24-h treatment of the cells with DHT resulted in ubiquitination and down-regulation of the EGFR. This effect could be inhibited by the anti-androgen flutamide, suggesting an androgen-dependent mechanism. On the other hand, DHT-treatment strongly increased AR levels in LNCaP cells. These data suggest a complex regulatory loop between activated AR and EGFR. In conclusion, activation of AR by both DHT and EGF/DHT involves the MAP kinase pathway. Long-term activation of AR results in increase of AR levels, which through so far unknown regulatory mechanisms results in ubiquitination and degradation of the EGFR.

Introduction

Prostate cancer is among the most frequently diagnosed cancers in males in the Western world (1). Although the molecular events involved in the development of prostate cancer are not well understood, there is general agreement that the androgen receptor (AR) plays a key role in the development, continued survival and proliferation of prostate cancer cells (2).

Proliferation and growth of androgen-dependent prostate cancer cells is mediated by the AR, a ligand-dependent nuclear receptor and transactivation factor. Binding of androgens such as dihydrotestosterone (DHT) to AR promotes the activation of protein kinases which phosphorylate several serine residues on AR. Some of these phosphorylations stabilize AR-homodimers, and the others influence trans-activation properties (3,4). The involvement of androgens in promotion of prostate tumour growth is explained by a generally accepted mechanism in which the ligand-bound receptor is phosphorylated, dimerised and translocated to the nucleus where it regulates the expression of genes involved in proliferation and survival (4). AR-positive prostate cancer is often treated by anti-androgenic therapy. However, presence of mutations on AR gene in prostate cancer cells may result in androgen-independent phenotype of the cells (5).

Involvement of epidermal growth factor receptor (EGFR) signal transduction pathway in pathogenesis, growth and metastasis in various cancers including prostate cancer is widely known (6-9). EGFR (ErbB1) belongs to the family of transmembrane receptor protein tyrosine kinases. The other members of the EGFR sub-family are ErbB2, ErbB3 and ErbB4. These receptors play a pivotal role in regulating cell proliferation, differentiation and transformation, and blocking of the EGFR pathway inhibits the proliferation of the cancer cells (10). Deregulation of EGFR has been reported to be involved in the transition of hormone dependent to hormone refractory state of prostate cancer cells (6). 'Cross-talk' of the EGFR signalling with AR has been postulated as a potential mechanism to activate AR in prostate cancer (11). EGFR activation results in downstream protein kinase C- δ (PKC- δ) signalling, representing a mechanism which is known to be critical for prostate cancer invasiveness (12). Two other important pathways, mitogen activated protein kinases (MAPK) and phosphatidylinositol 3-kinase (PI3K)/AKT, are also activated by EGFR (10,13), and

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Abbreviations: AR, androgen receptor; DHT, dihydrotestosterone; EGF, epidermal growth factor; EGFR, EGF receptor; ERK, extracellular signal-regulated kinase; FL, flutamide; MAPK, mitogen-activated protein kinase; MEK, MAP-ERK kinase; PI3K, phosphatidylinositol 3-kinase; PKC, protein kinase C; TGF, transforming growth factor

Key words: interaction of androgen receptor and EGF receptor, MAP kinase, ERK1/2, p38, EGFR ubiquitination, prostate cancer

activation of MAPK as an event depending on EGFR function in prostate cancer has been reported (13-15). Presence of EGF and transforming growth factor- α (TGF- α), the natural ligands and potent activators of EGFR, has been observed in many human tumours including prostate cancer (6,9,13,16,17). The reliance of prostate cancer pathogenesis on the activated EGFR and its downstream signalling pathways PI3K/AKT and/or MAPK is not completely elucidated (18). The major members of the MAPK family are ERK1/2 and p38. ERK1/2 contribute to cell differentiation, proliferation and survival whereas p38, a down-stream effector of PKC- δ , has been reported to promote apoptosis (19). However, many contradictory findings confound the roles of ERK and p38. ERK activation has been correlated with prostate cancer progression in line with Gleason score (20), while decline in ERK activities in advanced malignant prostate cancer has also been reported (21). Likewise, some authors (19,22) have suggested that p38 plays an important role in induction of apoptosis, while others (23) reported that the apoptotic rate in LNCaP cells increased significantly following p38 inhibition. Effects of p38, in particular, on prostate cancer cells are therefore complex (24). Further, reduction of EGFR protein levels has been observed in DU145-AR cells long-term treated with androgens (25). EGFR-stimulated intracellular signalling involving the MAPK pathway and the potential cross-talk between EGFR and AR demand further studies to elucidate the mechanism linking EGFR and AR signalling pathways in prostate cancer pathogenesis. The aim of the present study was to investigate the involvement of the EGFR-MAPK pathway in AR signalling in androgen-dependent prostate cancer, taking LNCaP cells as a model, and to clarify the mechanisms related to the modulation of EGFR protein expression in androgen treated condition.

Materials and methods

Reagents, antibodies and plasmid. The proteasome inhibitor MG-132, U0126, a specific inhibitor of MEK1/2, and the p38 inhibitor SB203580 were from Calbiochem (Merck Biosciences, Darmstadt, Germany). Protein A-sepharose beads were from Sigma (München, Germany). EGF was from R&D Systems GmbH (Wiesbaden, Germany), DHT and flutamide (FL) from Sigma (Steinheim, Germany). Antibodies used were against androgen receptor (AR) [AR(441): mouse antibody against amino acids 299-315 of AR of human origin (sc 7305 Santa Cruz Biotechnology, Heidelberg, Germany)]; EGFR [(Ab-1), mouse, Calbiochem]; phosphorylated p38 [pp38 (Thr180/Tyr182), rabbit, Cell Signaling Technology (Danvers, MA, USA)], p38 protein (New England Biolabs, Beverly, MA, USA); phosphorylated ERK1/2 and ERK1/2 proteins [pp44/42 and p44/42, rabbit, (Cell Signaling Technology)], β -tubulin (mouse, Upstate Biotechnology, Lake Placid, CA, USA), β -actin (mouse, Abcam, Cambridge, UK) and ubiquitin (rabbit, Dako, Hamburg, Germany). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were from Dianova (Hamburg, Germany). Amersham ECLplus Western Blotting detection reagent was from GE Healthcare (Freiburg, Germany), and radiographic film (Konika Minolta) was purchased from Hartenstein (Würzburg, Germany). The plasmid PLC0546A containing

luciferase under the control of androgen response element (AREluc) was described previously (26).

Cell culture and treatment procedure. LNCaP cells were grown for 7 days in phenol-red free RPMI-1640 medium (PAA Laboratories, Cölbe, Germany) supplemented with penicillin-streptomycin and 10% foetal calf-serum (FCS) stripped with dextran-charcoal (27). Before experimental treatments cells were kept overnight in the same medium supplemented with 2% stripped FCS.

Transfection. LNCaP cells (2×10^5 cells/well) were plated in RPMI - 10% stripped FCS in 6-well plates. Transfection of the plasmid PLC0546A, 0.05 μ g/well, was conducted the next day using effectene (Qiagen GmbH, Hilden, Germany) according to the procedure recommended by the manufacturer. After 24 h, the cells were washed with sterile PBS and RPMI containing 2% charcoal-stripped FCS was added. Another 24 h later, cells were used for further treatments.

Preparation of cell lysates and Western blot analysis. LNCaP cells were treated with DHT (1-5 nM), EGF (10 ng/ml), FL (1 μ M) and combinations thereof for 15 min. After treatment, the cells were washed with ice-cold phosphate-buffered saline (PBS) and scraped in PBS. The cell suspension was centrifuged and cell pellets were snap-frozen in liquid nitrogen. Thereafter, the pellets were suspended in lysis buffer (27) and incubated on ice for 30 min. The samples were then centrifuged at 13,000 \times g for 10 min at 4°C and the supernatants (lysates) were submitted to SDS-PAGE and Western blot analysis. Protein concentrations were determined using the DC Protein Assay kit from Bio-Rad (München, Germany). Some of the Western blot analyses were quantitatively evaluated using the ImageJ software (NIH, USA).

Measurement of luciferase activities. AREluc-transfected LNCaP cells were treated with DHT (5 nM), EGF (10 ng/ml), SB 203580 (20 μ M) or U0126 (20 μ M) and combinations thereof, or left untreated for controls. Luciferase activity was measured using the firefly luciferase reagents from Promega (Mannheim, Germany) and a Biolumat LB 9505 (Berthold, Bad Wildbad, Germany).

Immunoprecipitation and detection of ubiquitination of EGFR protein. LNCaP cells were treated with DHT (5 nM), FL (1 μ M), MG-132 (5 μ M), and combinations thereof, for 6 h. MG-132 was added 1 h prior to the other compounds. The EGFR protein was immunoprecipitated from the lysate with 2 μ g EGFR antibody for 12 h at 4°C using protein A sepharose beads. The immunocomplexes collected on the beads were washed with lysis buffer and were extracted in SDS loading buffer (10 mM Tris, pH 6.8, 2% SDS, 5% glycerol, 0.01% bromophenol blue, 0.35 M β -mercaptoethanol). After denaturation and centrifugation, the samples were separated in SDS-PAGE and used for Western blot analysis using ubiquitin antibodies and, after stripping of the membrane, EGFR antibodies.

Statistical analyses. The data were analysed statistically using paired t-tests with the help of Medcalc software (Medcalc

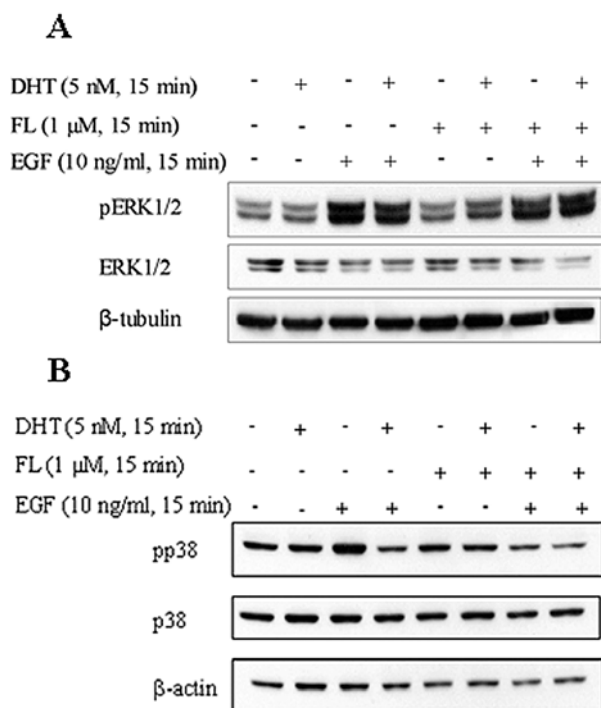


Figure 1. Influence of EGF, DHT and flutamide (FL) on MAPK pathway. LNCaP cells were treated with DHT, FL, EGF and combinations thereof. Untreated LNCaP cells were used as control. (A) Total protein (20 μ g) from each cell lysate was analysed by Western blotting with anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. β -tubulin was used to ensure equivalent loading of the gel incubating the same membrane with β -tubulin antibody after stripping. (B) Detection of p38 phosphorylation in the same lysates as shown in (A). As the loading control, anti- β -actin antibody was used.

Software, Mariakerke, Belgium). A $p < 0.05$ was considered as statistically significant.

Results

Effect of DHT treatment on ERK and p38 phosphorylation. We first evaluated the effect of 5 nM DHT short-term treatment (15 min) on activation of MAPK pathway by investigating the phosphorylation status of ERK1/2 and p38 in LNCaP cells. Treatment with 10 ng/ml EGF used as control resulted in strong phosphorylation of ERK1/2 and p38. DHT-treatment did not cause phosphorylation of ERK1/2 and p38 (Fig. 1). Interestingly, treatment of the cells with 5 nM DHT reduced EGF-induced p38 phosphorylation, and treatment with 1 μ M FL reduced the EGF-induced p38-phosphorylation below the basal phosphorylation level observed in untreated controls (Fig. 1B).

Effect of DHT and EGF on AR activity. We next explored the role of the activation of the MAPK pathway on AR activity. AR activation was measured by induction of firefly luciferase activity using AREluc as a reporter. Using DHT-treatment as control, we found a rapid 7-fold induction of luciferase activity after 3 h of treatment (Fig. 2A). The induction was transient, after 24 h DHT-treatment only 2-fold luciferase induction was measured (Fig. 2B). EGF alone had no effect on AR transactivation, however, a combination treatment of the cells with 5 nM DHT and 10 ng/ml EGF resulted in significantly stronger AR activation compared to DHT alone, about 40 and 15% higher luciferase activity were detected upon 3- and 24-h treatments, respectively. Both induction

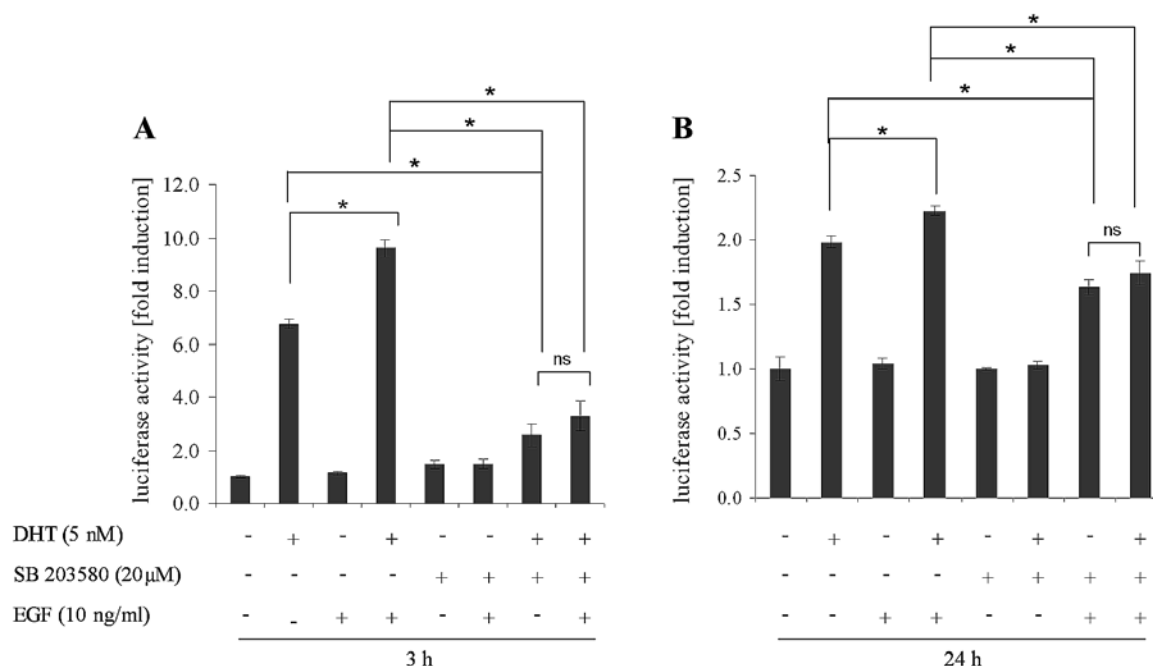


Figure 2. Influence of EGF-administration and p38-inhibition on DHT-induced ARE-dependent induction of luciferase in LNCaP cells. AR activation was measured by luciferase activity assays using AREluc as a reporter. Twenty-four hours after transfection with AREluc, LNCaP cells were treated with 5 nM DHT, 10 ng/ml EGF, 20 μ M SB203580 and combinations thereof, for 3 h (A) or for 24 h (B). Data are expressed as a fold induction of luciferase activity measured against the untreated AREluc transfected LNCaP cells. Data indicate mean \pm SD from two independent sets of experiments (four measurements). * $p < 0.05$; ns, statistically non-significant.

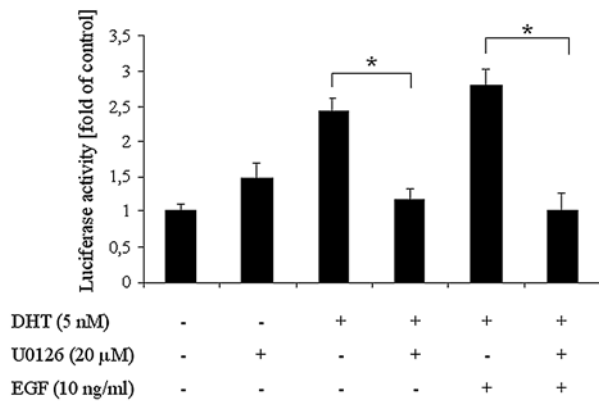


Figure 3. Influence of EGF-administration and MEK-inhibition on DHT-induced ARE-dependent induction of luciferase in LNCaP cells. AR activation was measured by luciferase activity assays using AREluc as a reporter. Twenty-four hours after transfection with AREluc, LNCaP cells were treated with 5 nM DHT, 10 ng/ml EGF and 20 μM U0126 and combinations thereof, for 24 h. Data are expressed as a fold induction of luciferase activity measured against the untreated AREluc transfected LNCaP cells. Data indicate mean ± SD from two independent sets of experiments (four measurements). * $p < 0.05$.

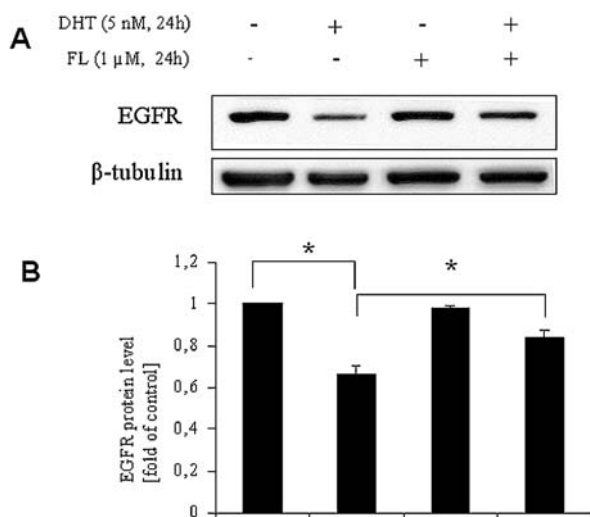


Figure 4. Reduction of EGFR protein levels by DHT-treatment in LNCaP cells. (A) Cells were treated with 5 nM DHT, 1 μM FL and DHT/FL for 24 h, and untreated cells were considered as control. EGFR protein was detected by immunoblotting. (B) Quantitative evaluation of immunoblots. EGFR protein level was presented as a fold of control. Data show mean ± SD of two independent sets of experiments. * $p < 0.05$.

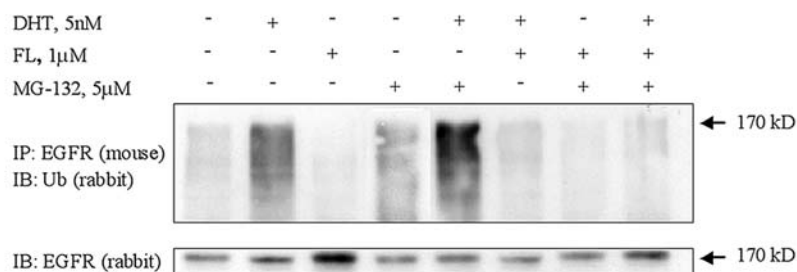


Figure 5. DHT induces polyubiquitination of EGFR protein. LNCaP cells were treated with 5 nM DHT, 1 μM FL, 5 μM MG-132 and combinations, for 6 h except MG-132 which was always added 1 h before the other compounds. EGFR protein was immunoprecipitated with EGFR antibody (mouse) and the precipitated protein was subjected to Western blot analysis with anti-ubiquitin antibody (Ub) (upper panel). The same blot was used to detect EGFR protein with anti-EGFR (rabbit) antibody after stripping (lower panel). The data are the representatives of two independent experiments.

of luciferase with DHT and DHT/EGF were significantly inhibited by the anti-androgen FL (not shown). The finding strongly claims that DHT and EGF synergistically induce AR signalling. Treatment of the cells with the specific p38-inhibitor SB 203580 caused significant reduction of both DHT- and DHT-EGF-induced luciferase activities after 3 and 24-h incubation (Fig. 2). This inhibition further indicates involvement of p38 MAPK in AR signalling.

To detect the role of ERK1/2 on AR-transactivation by DHT and EGF/DHT, U0126, a specific inhibitor of MEK which is an upstream regulator of ERK1/2, was used to inhibit phosphorylation of ERK1/2. Treatment of AREluc-transfected LNCaP cells with 20 μM U0126 reduced significantly DHT- and EGF/DHT-induced luciferase activities (Fig. 3). The results indicate interaction of ERK1/2 signalling with AR-transactivation. However, U0126 alone showed a small enhancement of luciferase activity as compared to control, which indicates that U0126 may affect AR-transactivation in LNCaP cells by other, so far unknown mechanisms.

These data suggest that EGF-activated MAPK pathway alone does not result in measurable AR activation in our cells, however, together with DHT, EGF shows a strong synergistic effect on AR activity.

DHT treatment reduces EGFR protein levels. Data presented so far suggest that activation of the EGFR and the EGFR-signalling pathway are related to AR activity. As long-term treatment with DHT was reported to reduce EGFR protein level in DU145-AR cells (25), we investigated the effect of DHT on EGFR in our experimental system. DHT treatment for 24 h was found to decrease EGFR levels significantly (Fig. 4) and the decreased levels of the protein were rescued by the anti-androgen FL. Therefore, to investigate the mechanism leading to decreased EGFR protein levels in presence of DHT, LNCaP cells were treated with 5 nM DHT, 1 μM FL, and the potent cell-permeable proteasome inhibitor MG-132 (5 μM) and combinations thereof. Incubation with MG-132 inhibits the proteasomal degradation of ubiquitin-conjugated proteins in mammalian cells. Since a report suggests that MG-132 provides maximal effect between 6-12 h (28), we accordingly selected 6-h treatment-duration. EGFR protein was immunoprecipitated from cell lysates and ubiquitination was detected with anti-ubiquitin antibody in Western blot analyses. Fig. 5 shows that in LNCaP cells, DHT treatment increased ubiquitination of EGFR markedly. Combination

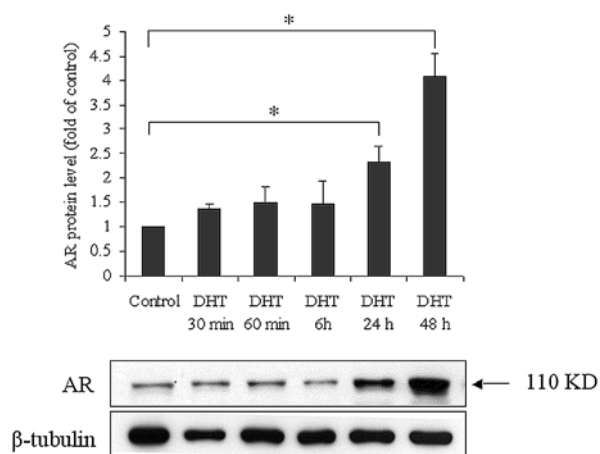


Figure 6. AR protein level in LNCaP cells at different time-points of DHT treatment. LNCaP cells were treated with 5 nM DHT for 30 min, 60 min, 6 h, 24 h and 48 h. AR was detected by Western blotting. β -tubulin was detected as loading control. Data indicate the mean \pm SD of two independent sets of experiments. * $p < 0.05$.

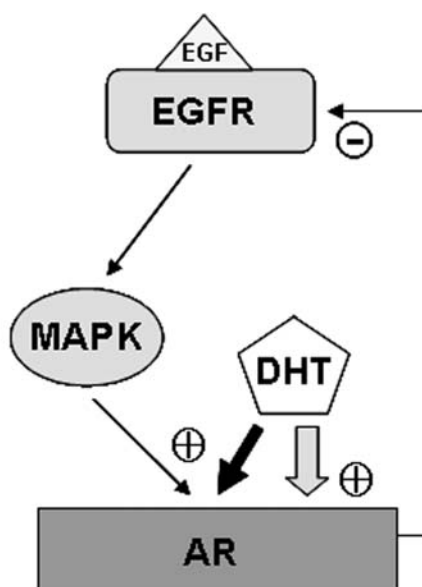


Figure 7. Model for the interaction of EGFR and AR signalling in prostate cancer cells. EGF and DHT synergistically activate the androgen receptor (black arrows). DHT-treatment, upon prolonged treatment, results in increase of AR protein levels (open arrow) and in decrease of EGFR levels.

treatment with DHT and MG-132 resulted in a stronger EGFR ubiquitination signal suggesting an accumulation of ubiquitinated EGFR due to inhibition of degradation. FL treatment reduced both basal EGFR ubiquitination and ubiquitination induced by DHT. The results clearly show that DHT enhances degradation of EGFR.

DHT treatment increases AR protein levels. To assess the role of DHT treatment on AR protein, LNCaP cells were treated with DHT (5 nM) for different the time-periods ≤ 48 h. The results showed that DHT treatment significantly increased AR protein levels 2.3- and 4-fold at 24 and 48 h, respectively (Fig. 6).

Discussion

The different types of MAPK have been suggested to be necessary for early processes in prostate cancer (24). ERK1/2 and p38 are the major members of the MAPK family. It has been widely claimed that ERK activation contributes to cell differentiation, proliferation and survival (29) and p38 activation promotes apoptosis (30). However, many conflicting reports (22-24) demand further studies regarding the role of MAPK in prostate cancer. The MAPK pathway links EGFR-mediated signals to nuclear events affecting cellular processes related to growth, mitosis, differentiation and death (31) and in prostate cancer, ErbB2 (HER2/neu) was reported to induce AR activation through the MAPK signalling cascade even without androgen stimulation (32). Therefore, we first investigated the effects of EGF alone and in combination with DHT treatment on phosphorylation of ERK1/2 and p38 and on activation of the androgen receptor in our experimental system. Although DHT-treatment alone did not induce ERK1/2 phosphorylation and EGF-treatment alone did not result in AR activation in LNCaP cells, there was a clear synergistic effect on AR activation upon combination treatment with DHT and EGF. The observation that the MEK inhibitor U0126 completely inhibited DHT- and DHT/EGF-induced AR activation clearly documents the role of ERK1/2 in DHT-induced AR activation.

The role of p38 seems to be more complex. Although treatment of the LNCaP cells with DHT resulted in marked inhibition of EGF-induced p38 phosphorylation, DHT/EGF-induced AR activity was significantly stronger as compared to treatment with DHT or EGF, respectively. While p38 may be dephosphorylated (inactivated) in conditions resulting in AR activation, inhibition of this enzyme by SB 203580 surprisingly resulted in inhibition of AR activation by DHT. This paradoxical finding suggests complex mechanisms of interaction of p38 with AR function. Although p38 was claimed as apoptotic inducer by some authors (19,22,33), our findings agree with reports that p38 is directly involved in AR-mediated cell proliferation, survival and growth since AR transactivation results in cell proliferation in LNCaP cells (4).

The observation of a synergistic effect of DHT and EGF on AR activity is supported by the findings of Jones *et al* (34) who reported that combined treatment with EGF and 5 α -DHT produced an additive effect on cell proliferation. However, the molecular basis of the interactions between DHT and EGF in prostate cancer remains unclear. It has been reported that androgen regulates EGFR receptor expression in endometrial cells (35) and DHT inactivates prostatic acid phosphatase which causes stimulation of EGF signal pathway (36,37). Thus, the molecular basis of EGF/DHT synergism may be a future topic of interest.

The role of androgen in the control of AR protein levels as available in the literature is conflicting. Upon androgen treatment, an increase in total AR protein content (38), post-translational modification without alteration at the AR protein level (39) or stabilization of the AR protein (40,41) have been reported. Yeap *et al* showed that DHT decreased total AR mRNA, but increased AR protein (42). Androgen-mediated negative autoregulation of AR mRNA has been

reported by many authors (38,39,43), and androgen-induced AR mRNA transcription has been suggested to increase paradoxically AR mRNA stability (39), which in turn might lead to increased AR protein in LNCaP cells exposed to DHT for 24 h and more. Although we did not study AR mRNA levels, we found that DHT-treatment of LNCaP cells for 24 h and 48 h resulted in significant increase of AR protein levels.

It was reported that prolonged treatment of prostate cancer cells with androgens results in down-regulation of EGFR protein levels. Incubation with 1 nM DHT for 15 days reduced the EGFR protein in DU145-AR cells (DU145 prostate cancer cells stably transfected with AR cDNA) (25). This agrees with our findings where treatment with 5 nM DHT for 24 h reduced EGFR protein significantly in LNCaP cells. The DHT-dependence of down-regulation of the AR protein is supported by the observation that the anti-androgen FL was able to rescue EGFR protein. We conclude that EGFR protein down-regulation is the result of exposure of the cells to DHT. This finding may be of importance for therapy of androgen-dependent prostate cancer. Anti-androgenic therapy may result in maintenance of the EGFR signalling properties of the cancer cells and hence in sustained stimulation of proliferation by EGF or TGF α .

Finally, we were interested in the mechanism involved in DHT mediated down-regulation of EGFR protein in LNCaP cells. In our experiment polyubiquitination of EGFR protein was found increased in immunoprecipitates from DHT-treated cells, and especially from MG-132 and DHT/MG-132 treated cells. This suggests proteasomal degradation of EGFR as a result of prolonged androgen exposure. However, it was reported, that EGFR as such is not a target for proteasomal degradation (44), but ubiquitinated EGFR is removed from the cells via endocytosis and subsequent degradation in lysosome (45). MG-132 has further been reported to decrease transcriptional activity of AR by eliminating androgen-induced nuclear translocation and co-activator recruitment (46,47). Thus, DHT-mediated polyubiquitination and proteasomal degradation of EGFR might be controlled through mechanism(s) other than increased AR level. However, although it is not known whether the DHT-related increased AR protein levels are involved in the process of EGFR degradation, it may be concluded that prolonged exposure of LNCaP cells to DHT activates a regulatory loop that modulates cellular EGFR levels.

In conclusion, our results suggest a complex regulatory loop between AR and EGFR function and expression levels in AR-positive prostate cancer cells. Fig. 7 summarizes the findings in a simple model. We suggest that both ERK1/2 and p38 MAP kinases are involved in androgen-dependent AR activation in LNCaP cells and EGF and DHT show a synergistic positive effect on AR transactivation. Long-term AR activation increases the AR protein level and decreases the EGFR protein level.

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