

Interruption of Nuclear Factor κ B Signaling by the Androgen Receptor Facilitates 12-*O*-Tetradecanoylphorbolacetate-Induced Apoptosis in Androgen-sensitive Prostate Cancer LNCaP Cells

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ABSTRACT

12-*O*-tetradecanoylphorbolacetate (TPA) influences proliferation, differentiation, and apoptosis in a variety of cells including prostate cancer cells. Here, we show that androgen treatment potentiates TPA-induced apoptosis in androgen-sensitive prostate cancer LNCaP cells but not in androgen-independent prostate cancer cell lines DU145 and PC-3. The use of the antiandrogen bicalutamide (Casodex) rescued LNCaP cells from 5- α -dihydrotestosterone (DHT)/TPA-induced apoptosis, suggesting that DHT/TPA-induced apoptosis is mediated by androgen/androgen receptor (AR). In addition, a caspase-3 inhibitor (Ac-DEVD-CHO) reduced the level of apoptosis, suggesting that DHT/TPA-mediated apoptosis occurs through a caspase-3-dependent pathway. A functional reporter assay using nuclear factor (NF) κ B-luciferase and an electromobility gel shift assay showed that DHT suppressed NF κ B activity. In addition, apoptosis mediated by combined DHT/TPA treatment was abrogated by overexpression of the NF κ B subunit p65 in LNCaP-p65 cells, suggesting that NF κ B may play an important role in regulating the effects of androgen/AR and TPA on apoptosis. Furthermore, use of the c-Jun N-terminal kinase (JNK) inhibitor SB202190 showed that the combination of DHT/TPA increased JNK activation in LNCaP cells but not in LNCaP-p65 cells, demonstrating that NF κ B may be able to suppress JNK activity. These results indicate that androgen/AR facilitates TPA-induced apoptosis by interruption of the NF κ B signaling pathway, leading to activation of JNK in LNCaP cells. These data describe a signaling pathway that could potentially be useful in proposed therapeutic treatment strategies exploiting combinations of different agents that control apoptosis in prostate tumors.

INTRODUCTION

One characteristic of prostate cancer is the initial dependence of tumor growth on circulating androgens, and androgen-ablative pharmacotherapy is a common treatment strategy for prostate cancer (1, 2). Androgen deprivation results in the involution of prostate tissue, a process dependent in part on apoptosis of prostate epithelial cells (3). However, androgen-independent tumor cells that are unresponsive to androgen deprivation eventually arise, and the progression of tumor cells to complete androgen independence is associated with a poor prognosis (4). As dependency on androgen is lost, other growth factor signaling pathways must become activated to enable the survival and growth of androgen-independent tumors. Understanding the development of androgen-independent prostate tumor cells and devising treatment schemes for targeting these cells, such as intermittent androgen therapy, would provide therapeutic options currently not available for advanced prostate cancer.

Intermittent androgen therapy has been proposed as a means to minimize adverse effects associated with androgen ablation therapy. Repeated cycles of intermittent treatment may postpone the emergence of androgen-independent tumor cells, decrease the severity of advanced treatment-related effects, and be less costly than continuous androgen-ablative therapy. It has been hypothesized that reintroduction of androgen after a period of androgen deprivation may alter the growth behavior of the remaining tumor cells (5, 6), an effect that may be caused, in part, by alterations in the apoptotic potential of remaining tumor cells in the presence of emergent androgen levels. The effect of intermittent androgen therapy has been studied in animals, but the molecular mechanisms controlling the delay to androgen independence are poorly understood. Because apoptosis may play a role in such processes, an understanding of the contribution of androgen/AR² to apoptosis induction is important in developing a rationale for exploitation of such therapeutic approaches.

Characterization of the role of AR in controlling apoptosis and the participation of AR signaling in cross-talk with other pathways will clarify the role of androgen in prostate tumor growth. Induction of apoptosis is determined by the integration of numerous signals that control entry into the cell death pathway. One pathway that controls cell growth and differentiation is activation of PKC (7, 8). Activation of PKC by TPA induces apoptosis in the androgen-sensitive cell lines LNCaP (9) and HaCaT (10) but not in the androgen-independent cell lines PC-3 and DU145 (9), or in normal keratinocytes (10). Another pathway involved in growth control and suppression of apoptosis involves NF κ B, which is a member of the nuclear transcription factor family of proteins (11). NF κ B consists of dimers of Rel family proteins (12, 13), each of which contains a conserved Rel homology domain that allows dimerization and a DNA binding domain that allows binding to NF κ B response elements on the target genes. In mammalian cells, there are five members of the NF κ B family: NF κ B1 (p50/p105), NF κ B2 (p52, p100), RelA (p65), cRel, and RelB (12). NF κ B is retained in the cytoplasm in an inactive form by the inhibitor protein I κ B (14, 15). In response to certain extracellular signals such as TNF, interleukin-1, TPA, or lipopolysaccharide (16, 17), IKK is activated and phosphorylates I κ B, releasing NF κ B, which then translocates to the nucleus and binds to DNA.

It has been reported that NF κ B induces expression of target genes that contribute to tumor progression. These genes include immunoregulatory, inflammatory, and antiapoptotic genes as well as genes that regulate cell proliferation (18). In the androgen-independent prostate cell lines PC-3 and DU145, the activity of NF κ B is high compared with that in the androgen-dependent prostate cell line LNCaP (19). It has also been shown that in LNCaP cells cross-talk occurs between NF κ B signaling pathways and steroid receptor sig-

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²The abbreviations used are: AR, androgen receptor; PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol acetate; NF κ B, nuclear factor κ B; TNF, tumor necrosis factor; IKK, I κ B kinase; JNK, c-Jun N-terminal kinase; DHT, 5- α -dihydrotestosterone; TUNEL, terminal transferase-mediated dUTP-biotin nick end-labeling; Luc, luciferase; EMSA, electromobility gel shift assay.

naling pathways, such as glucocorticoid receptor and AR pathways (20–22). Differences in NF κ B activity between androgen-sensitive and androgen-independent prostate cancer cell lines may contribute to androgen independence (23).³ Moreover, when cells undergo apoptosis, JNK activity is increased (24, 25). Recent studies have shown that cross-talk exists between the JNK and NF κ B signaling pathways (25, 26). It has been proposed that JNK activity is an upstream event relative to activation of caspase cascades during apoptosis.

The differences between androgen-sensitive and androgen-independent cell lines with respect to TPA-induced apoptosis and NF κ B activity (23) prompted the hypothesis that TPA may induce apoptosis through an AR-dependent pathway in LNCaP cells. To define the role of androgen/AR in apoptosis signaling in LNCaP cells, we used a regimen of treatment with androgen for 24 h, followed by 24-h treatment with 1 nM TPA. To characterize the response of LNCaP cells to these treatment conditions, we determined the percentage of cells undergoing apoptosis and the activities of AR, NF κ B, caspases, and JNK in the apoptotic cascade. Our data show that interruption of NF κ B signaling by androgen/AR facilitates TPA-induced apoptosis via activation of JNK. This observation suggests that androgen pretreatment sensitizes cells to the apoptotic effect of PKC activation in a prostate cancer cell line. Resumption of androgen levels during intermittent androgen therapy may sensitize tumor cells to proapoptotic agents, providing an opportunity to improve the effectiveness of intermittent androgen therapy.

MATERIALS AND METHODS

Cell Culture, Plasmids, and Reagents. The human prostate cancer LNCaP cell line was maintained in RPMI 1640 containing 10% FCS, penicillin (25 units/ml), and streptomycin (25 μ g/ml). DHT and TPA were purchased from Sigma Chemical Company. Caspase inhibitors Z-VAD-fmk and DEVD-cho were purchased from Calbiochem. The anti-AR polyclonal antibody NH27 was produced as described previously (27). Anti-I κ B α and Anti-Rel (p65) were obtained from Santa Cruz Biotechnology. A monoclonal antiactin antibody was obtained from Amersham Biosciences. The plasmids pCDNA3, pCDNA3-RelA, pCMV, pCMV-mIkB α (Ser32–36), and pCMV-NF κ B Luc reporter gene were gifts from Dr. Edward M. Schwarz (University of Rochester, Rochester, NY).

Detection of Apoptosis. To visualize apoptotic nuclei, LNCaP cells were fixed in 4% paraformaldehyde (pH 7.4) and subjected to the TUNEL assay. End-labeled DNA was visualized using FITC-conjugated avidin, and total cell count was determined by 4',6-diamidino-2-phenylindole staining. In brief, myocytes were incubated for 1 h at 37°C in terminal deoxynucleotidyltransferase buffer containing 140 mM sodium cocodylate, 1 mM cobalt chloride, 30 mM Tris-HCl (pH 7.2), 50 units of terminal deoxynucleotide transferase, and 1 nmol of fluorescein-conjugated dUTP (Roche Molecular Biochemicals). After the terminal deoxynucleotidyltransferase reaction, cells were washed three times in PBS and mounted on glass slides. The number of FITC-labeled cells per 100 4',6-diamidino-2-phenylindole-stained cells was used as a measure of the percentage of apoptosis. Genomic DNA was isolated for nucleosomal DNA fragmentation by gel electrophoresis, as described previously (28).

Transfections and Reporter Gene Assays. Transfections, using the calcium phosphate precipitation method, and Luc assays were performed as described previously (29). Briefly, $1-4 \times 10^5$ cells were plated on 35- or 60-mm dishes 24 h before adding the precipitation mix containing an NF κ B-Luc reporter gene. In each experiment, the total amount of transfected DNA/dish was maintained at a constant level by the addition of an empty expression vector (pCMV). The medium was changed 24 h after transfection, and the cells were treated with 1 nM DHT for 24 h, followed by treatment with TPA for another 16 h. The cells were then harvested, and whole cell extracts were used for the Luc assay. Luc activity was determined using a Dual-Luciferase Reporter Assay System (Promega) and measured with a luminometer.

Western Blot Analysis. LNCaP cells were treated with 10 nM DHT for 24 h, followed by 24 h of treatment with 1 nM TPA. The medium was removed, and the attached cells were washed with PBS. Proteins were extracted by cell lysis with SDS, and protein concentrations were measured with the BCA protein reagent (Pierce Chemical Co., Rockford, IL). Equal amounts of total protein (50 μ g) were loaded and run on a 10% SDS-polyacrylamide stacking gel with a Tris/glycine running buffer system and then transferred to a polyvinylidene difluoride membrane (0.2 μ m) in a mini electrotransfer unit (Bio-Rad, Hercules, CA). The blots were probed with anti-AR, anti-RelA, and antiactin antibodies. Immunoblot analysis was performed with horseradish peroxidase-conjugated antirabbit and antimouse IgG antibodies using enhanced chemiluminescence Western blotting detection reagents (Amersham Biosciences).

EMSA. Nuclear extracts of cells were prepared as described previously (30). ³²P-radiolabeled duplex oligonucleotide probes containing the NF κ B consensus binding site 5'-AGTTGAGGGGACTTTCCAGGC-3' and the mutant sequence 5'-AGTTGAGGCGATTTCCAGGC-3' were used as templates for EMSA experiments (Santa Cruz Biotechnology). DNA binding reaction mixtures (20 μ l) were prepared on ice and contained 10 μ g of nuclear extract, 2 μ g of double-stranded probe, poly(dI-dC) (Amersham Pharmacia Biotech), and 10 μ g of BSA in 20 mM HEPES (pH 7.9), 5% glycerol, 1 mM EDTA, and 5 mM DTT. Nuclear protein complexes were resolved on a native 5% polyacrylamide gel in 1 \times Tris-borate EDTA (pH 8.0) and detected by autoradiography (30).

JNK Assay. For the JNK kinase assay, subconfluent LNCaP cells were incubated for 24 h in RPMI medium containing 10% charcoal dextran-fetal bovine serum and then stimulated with 10 ng/ml TNF- α for 15 min. Total cell lysates were prepared as described previously (31), and JNK activity was determined using an anti-phospho-JNK antibody and JNK assay kit according to the manufacturer's instructions (New England Biolabs). Briefly, GST-c-Jun (aa 1–89) fusion protein bound to glutathione-Sepharose beads was incubated with cell lysates for 2 h at 4°C and then centrifuged at 15,000 rpm for 15 min to pull down JNK. The samples were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The kinase activity was determined by Western blot analysis of phosphorylated c-Jun using a rabbit anti-phospho-c-Jun antibody.

RESULTS

AR Facilitates TPA Induction of Apoptosis in LNCaP Cells.

Previous studies have shown that 10 or 100 nM TPA are able to induce apoptosis in the androgen-sensitive cell line LNCaP but not in the androgen-independent cell lines PC-3 and DU145 (31, 32), suggesting that androgen/AR signaling may be involved in TPA-induced apoptosis in LNCaP cells. To confirm the previous findings in our experimental system, we first treated LNCaP cells with either vehicle (ethanol; Fig. 1Aa) or 10 nM DHT (Fig. 1Ab) for 24 h and then treated with 1 nM TPA for another 24 h (Fig. 1A, c and d). Cells were then observed under light microscopy and photographed.

Treatment for 24 h with 10 nM DHT alone (Fig. 1Ab) or 1 nM TPA alone (Fig. 1Ac) does not induce apoptosis. However, treatment with 10 nM DHT for 24 h, followed by 24-h treatment with 1 nM TPA induced substantial apoptosis as determined by cell morphology (Fig. 1Ad) and DNA fragmentation (Fig. 1B, Lane 4). The percentage of cells undergoing apoptosis was determined by TUNEL assay, as described in "Materials and Methods" and shown in Fig. 2A. These results indicate that androgen/AR enables a lower concentration of TPA to induce apoptosis in LNCaP cells.

In agreement with other studies, our data show that neither 1 nM TPA alone nor the combination of 10 nM DHT and 1 nM TPA are able to induce apoptosis in the androgen-independent, AR-negative cell lines PC-3 and DU145 (Fig. 2A; Refs. 9, 31). To show that TPA-induced apoptosis in LNCaP cells is dependent on signaling through the AR, we used the AR antagonist bicalutamide (Casodex) to block the effect of AR signaling. Pretreatment of cells with 1 μ M bicalutamide (Casodex) significantly reduced apoptosis induced by the com-

³ S. Altuwajiri, unpublished results.

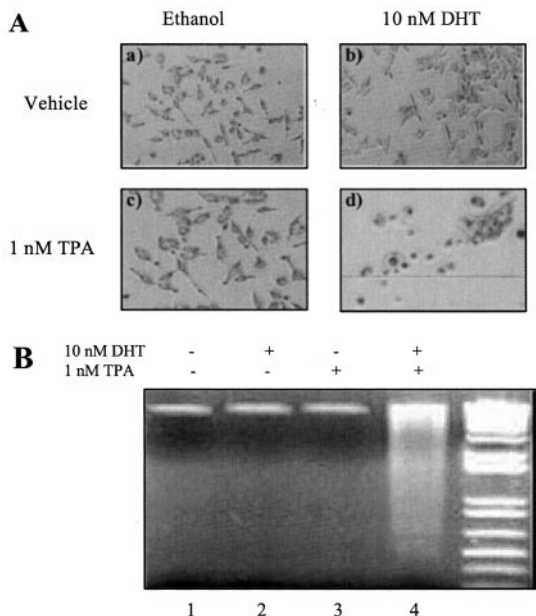


Fig. 1. A, treatment with 10 nM DHT for 24 h, followed by 1 nM TPA for 24 h, induced substantial apoptosis of LNCaP cells. LNCaP cells were treated with ethanol (a and c) or 10 nM DHT (b and d) for 24 h and then treated with 1 nM TPA (c and d) and observed by light microscopy. B, electrophoretic analysis of DNA extracted from LNCaP cells after treatment with ethanol (Lanes 1 and 3) or 10 nM DHT (Lanes 2 and 4) for 24 h, followed by 1 nM TPA for 24 h (Lanes 3 and 4). Cells were harvested and fixed in ethanol, and DNA was extracted. The DNA extracts from cells were resolved by electrophoresis on 1.2% agarose gels. Migration of the DNA marker is indicated on the right.

combination of TPA and DHT, as shown in Fig. 2B. The ability of bicalutamide to prevent >70% of the DHT/TPA-induced apoptotic effect suggests that AR is involved in DHT/TPA-induced apoptosis.

DHT/TPA-induced Apoptosis in LNCaP Cells Is Caspase-3 Dependent. A number of studies have shown that exposure to radiation in the presence of TPA enhances caspase-dependent apoptosis (8, 9, 33). We tested whether apoptosis induced by the combination of DHT and TPA involves activation of the apoptotic caspase cascade. Caspase-3 plays a major role in apoptosis, and detection of the activated form of caspase-3 represents apoptotic activity within the cell. We used zVAD-fmk, a general caspase inhibitor, and Ac-DEVD-CHO, a caspase-3 inhibitor, to show the role of caspase activation in the observed apoptotic response. Cells were treated with 10 nM DHT for 24 h and then incubated with either zVAD-fmk or Ac-DEVD-CHO for 30 min, followed by 24-h treatment with 1 nM TPA. Cells were then assayed for apoptosis using a TUNEL assay. The results show that apoptosis was reduced at least 50% by zVAD-fmk and 60% by Ac-DEVD-CHO (Fig. 3A), suggesting DHT/TPA-mediated apoptosis is a caspase-3 dependent pathway.

To confirm that the observed apoptosis was associated with caspase activation, we used Western blot analyses to detect levels of caspase-3 and its active cleavage product. The combination of DHT and TPA increases caspase-3 activity as seen in the cleavage of the pro-caspase-3 (M_r 37,000) into two fragments of M_r 17,000 and M_r 12,000 (Fig. 3B, Lane 4). These data show that the majority of apoptosis generated by the combination of DHT before treatment and TPA is caspase-3 dependent.

AR Interrupts NFκB Signaling in LNCaP Cells. It is known that TPA is a potent activator of IKK (34–36). It is also known that NFκB is capable of protecting cells from apoptosis (37). In the androgen-independent prostate cell lines PC-3 and DU145, the activity of NFκB is higher compared with the androgen-sensitive prostate cell line LNCaP (19). It also has been shown that cross-talk between NFκB signaling pathways and steroid receptor signaling pathways, such as

those of the glucocorticoid receptor and AR, occurs in LNCaP cells (20–22). To determine the effect of treatment with 10 nM DHT, followed by 1 nM TPA, on NFκB activity, we performed a transactivation assay using a Luc reporter construct responsive to active NFκB. Treatment with 1 nM TPA alone shows significant activation of NFκB (Fig. 4A, Lane 3). Pretreatment with 10 nM DHT abrogated this activation (Fig. 4A, Lane 4), indicating an interaction between AR signaling and TPA-induced NFκB activation.

To further establish the effects of DHT on TPA-induced NFκB activation, we performed EMSA. The results show that 1 nM TPA activated NFκB (Fig. 4B, Lane 1) and that pretreatment with 10 nM DHT decreased the amount of binding activity (Fig. 4B, Lane 2). Expression of the IκB-α protein has been used as a marker for the activity of NFκB. Therefore, we performed Western blot analyses on protein extracts from treated LNCaP cells to determine the levels of IκB-α under various treatment conditions, as shown in Fig. 4C. Treatment with 10 nM DHT alone caused an increase in the expression of IκB-α protein, suggesting that AR might play a role in regulating IκB-α expression (Fig. 4C, Lane 2). IκB-α protein expression decreased after 1 nM TPA treatment (Fig. 4C, Lane 3) and was restored when the cells were pretreated with 10 nM DHT (Fig. 4C, Lane 4). Western blot analyses of AR expression shows that DHT treatment increases AR protein expression (Fig. 4C, Lanes 2 and 4) and that TPA treatment alone had no effect on the AR level (Fig. 4C, Lane 3). Taken together, these results suggest that androgen suppresses NFκB activity (Fig. 4, B, Lane 2, and C, Lanes 2 and 4).

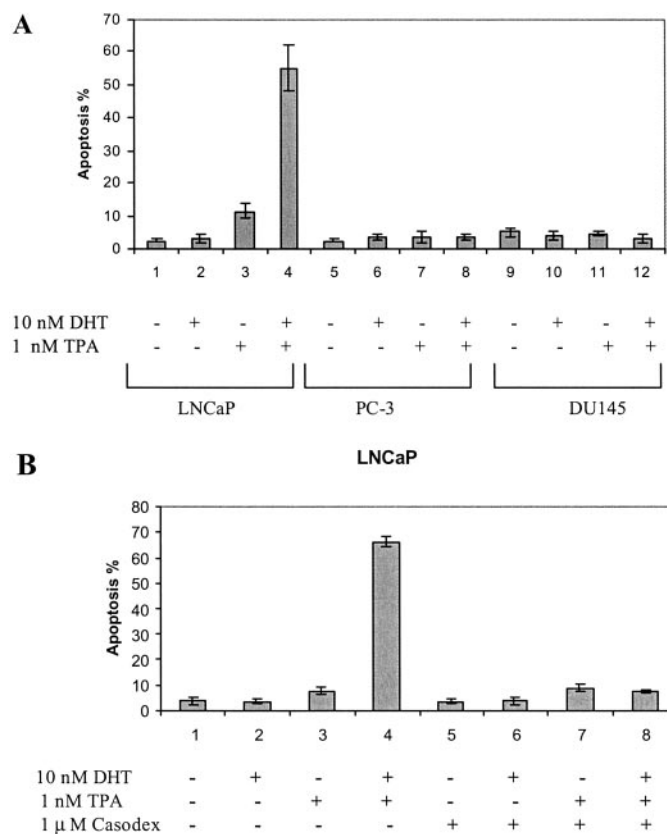


Fig. 2. Effect of the combination of 10 nM DHT and 1 nM TPA on androgen-independent prostate cell lines. A, LNCaP, PC-3, and DU145 cells were treated with ethanol (Lanes 1, 3, 5, 7, 9, and 11) or 10 nM DHT (Lanes 2, 4, 6, 8, 10, and 12) for 24 h and then with 1 nM TPA for 24 h (Lanes 3, 4, 7, 8, 11, and 12). Cells were harvested, and the level of apoptosis was determined by TUNEL assay. B, LNCaP cells were treated with ethanol (Lanes 1, 3, 5, and 7) or 10 nM DHT (Lanes 2, 4, 6, 7, and 8), with or without bicalutamide (Casodex; Lanes 5, 6, and 8) for 24 h, followed by treatment with 1 nM TPA for 24 h (Lanes 3, 4, 7, and 8). Cells were harvested and the level of apoptosis was determined by TUNEL assay.

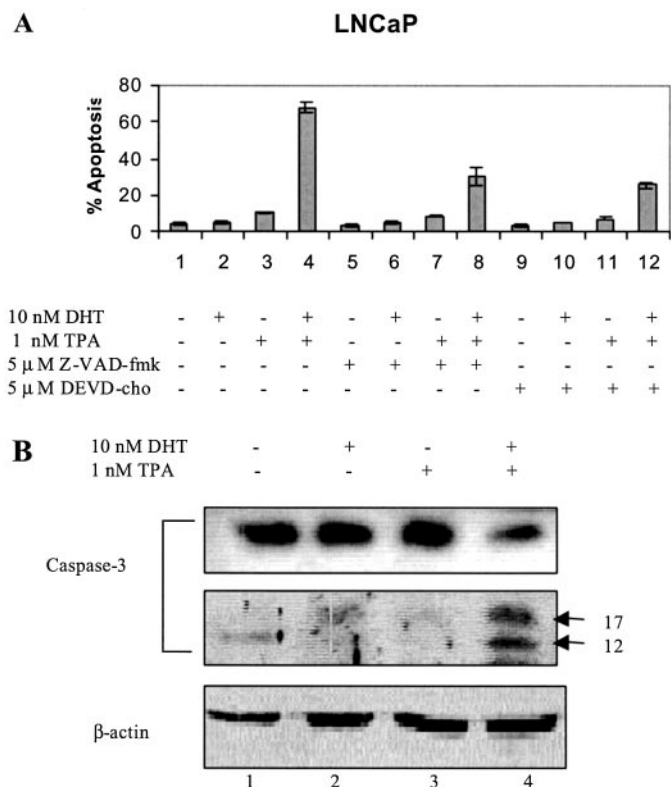


Fig. 3. The combination of DHT/TPA-induced apoptosis in LNCaP cells is caspase-3 dependent. **A**, LNCaP cells were treated with ethanol (Lanes 1, 3, 5, 7, 9, and 11), 10 nM DHT (Lane 2, 4, 6, 8, 10, and 12), 10 μM Z-VAD-fmk (Lanes 5-8), or 10 μM DEVD-cho (Lanes 9-12) for 24 h, followed by treatment with 1 nM TPA (Lanes 3, 4, 7, 8, 11, and 12). Apoptosis was detected by TUNEL assay. **B**, LNCaP cells were treated with ethanol (Lanes 1 and 3) or 10 nM DHT (Lanes 2 and 4) for 24 h, followed by treatment with 1 nM TPA (Lanes 3 and 4). Expression levels of caspase-3 were determined by Western blot with a polyclonal antibody against caspase-3. Equal amounts of lysates were resolved by 10% SDS-PAGE. The arrows indicate the active subunits of caspase-3 after activation.

Ectopic Expression of RelA Protects LNCaP Cells from Androgen/AR and TPA-induced Apoptosis. To investigate further the role of NFκB in DHT/TPA-induced apoptosis, we hypothesized that overexpression of NFκB would protect cells from apoptosis induced by DHT/TPA treatment. LNCaP cells were transfected with the pCDNA3 vector or with pCDNA3 containing the major activation subunit of NFκB, known as RelA p65. Several independent transfected cell lines were established, and protein extracts from each were subjected to Western blot analyses with anti-p65 (RelA) antibody (Fig. 5A). The clonal line LNCaP-RelA (6) was chosen for additional analysis.

LNCaP-RelA (6) cells were treated with 10 nM DHT for 24 h, followed by 24-h treatment with 1 nM TPA. Compared with vector-only transfected LNCaP cells, the LNCaP-RelA (6) cells were protected from apoptosis as determined by TUNEL staining (Fig. 5, B and C). These results indicate that overexpression of RelA protects cells from apoptosis induced by the combination of DHT and TPA.

Induction of Apoptosis by Androgen/AR and TPA Is Mediated by JNK Activity. Reports have shown that when cells undergo apoptosis, JNK activity is increased (24, 25), and recent studies have demonstrated that cross-talk exists between the JNK and NFκB signaling pathways (25, 26). It has also been proposed that JNK activity is an upstream event relative to the activation of caspase cascades during apoptosis. Because of the relationship between JNK and NFκB, we tested whether the combination of DHT and TPA has an effect on JNK activation in LNCaP cells. The p38/JNK inhibitor SB202190 blocks both p38 and JNK activation (26). SB202190 was used to determine the effect of 10 nM DHT treatment, followed by 1

nm TPA, on JNK activity. Fig. 6, A and B, shows the suppressive effect of SB202190 on TPA-induced apoptosis after DHT pretreatment. Inhibition of p38/JNK by SB202190 results in a >50% decrease in the induction of apoptosis as shown by TUNEL assay (Fig. 6B). These experiments suggest that induction of apoptosis in LNCaP cells by DHT/TPA is mediated through JNK activity. Western blot analyses of protein extracts, using an antibody for the activated (phosphorylated) form of JNK, show an increase in the detectable level of activated JNK in treated LNCaP cells (Fig. 6C, Lane 4) but not in LNCaP-RelA cells (Fig. 6C, Lane 8), suggesting that NFκB may be able to suppress JNK activity.

To determine whether activation of JNK activity is linked to suppression of NFκB activity, we used inhibitors of NFκB. Fig. 7A shows the effect of NFκB inhibitors on NFκB Luc activity in a transactiva-

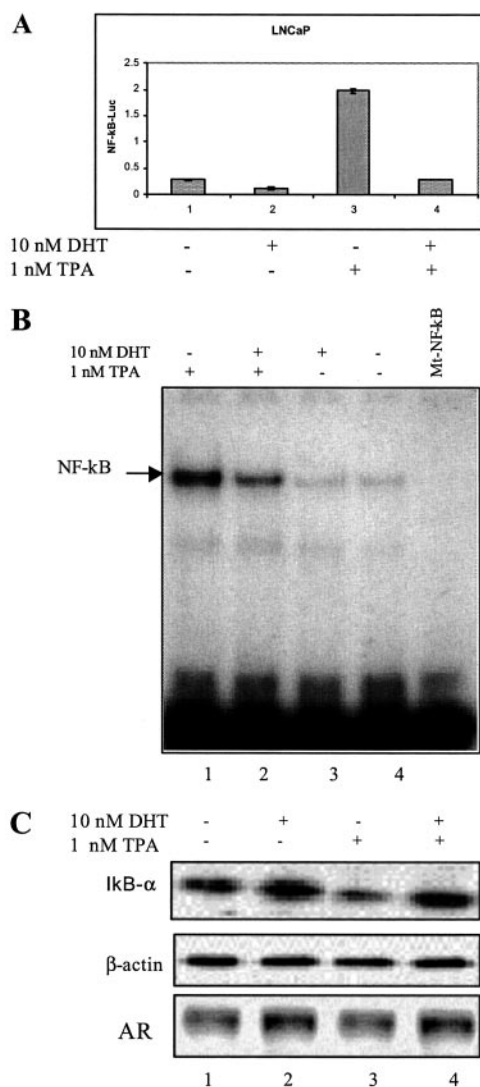


Fig. 4. AR interrupts NFκB signaling in LNCaP cells. **A**, TPA-induced NFκB transactivation is repressed by pretreating LNCaP cells with 10 nM DHT for 24 h. Cells were transfected with pNF-kB-Luc for 24 h before treatment. **B**, EMSA experiments were performed using protein samples from LNCaP cell nuclear extract cell. Extracts (5 μg) were incubated with ³²P-labeled wild-type NFκB or mutant oligomers (Lane 5). LNCaP cells were treated with ethanol (Lanes 1 and 3) or 10 nM DHT (Lanes 2 and 4) for 24 h, followed by treatment with 1 nM TPA for 24 h (Lanes 3 and 4). **C**, expression levels of IκBα were determined by Western blot analysis with monoclonal antibody against IκBα. LNCaP cells were pretreated with ethanol (Lanes 1 and 3) or 10 nM DHT (Lanes 2 and 4), for 24 h, followed by treatment with 1 nM TPA for 24 h (Lanes 3 and 4). Expression levels of AR were determined by Western blot analysis with a monoclonal antibody against AR. LNCaP cells were treated with ethanol (Lanes 1 and 3) or 10 nM DHT (Lanes 2 and 4) for 24 h, followed by treatment with 1 nM TPA for 24 h (Lanes 3 and 4).

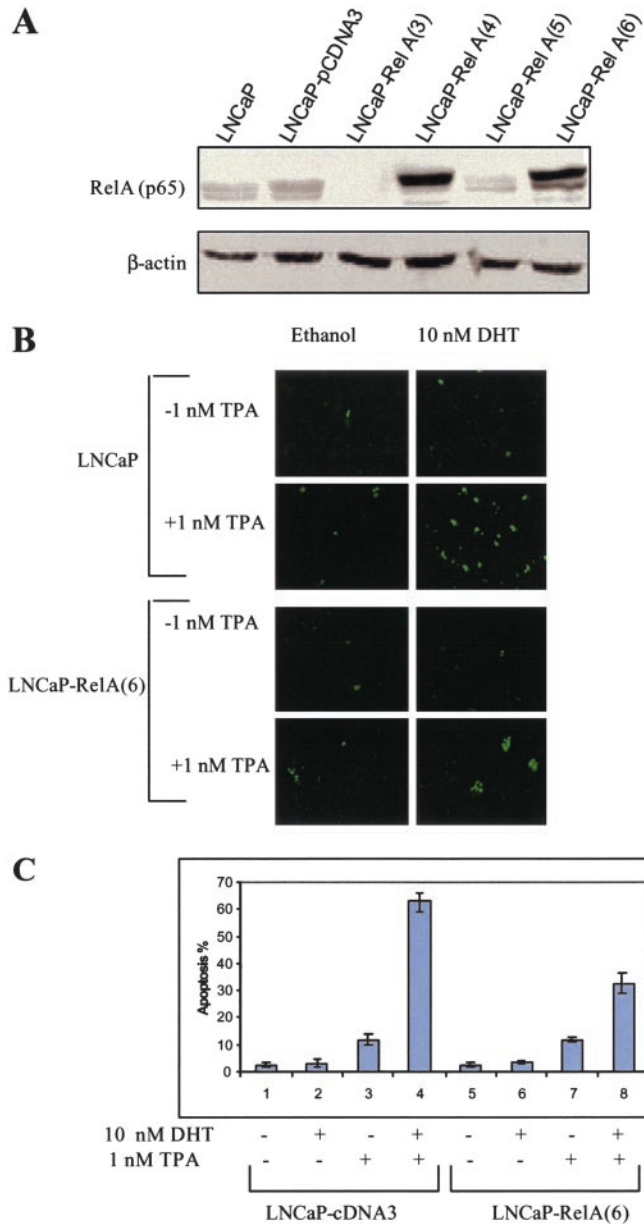


Fig. 5. Ectopic expression of RelA protects LNCaP cells from androgen/AR and TPA-induced apoptosis. A, Western blot analysis of selected clones of LNCaP cells transfected with pCDN3-RelA. Expression levels of RelA proteins were analyzed by Western blot with a monoclonal antibody against RelA-NF-κB. LNCaP (Lane 1), LNCaP-cDNA3 (Lane 2), and LNCaP-RelA clones 3–6 (Lanes 3-6) were analyzed. B, *in situ* apoptosis assay by detection of fragmented DNA strands. TUNEL- and Hoechst 33258-positive cells were photographed with the use of an Olympus light microscope equipped with an epifluorescence system. C, LNCaP and LNCaP-RelA cells were treated with ethanol (Lanes 1, 3, 5, and 7) or 10 nM DHT (Lanes 2, 4, 6, and 8) and then with TPA for 24 h (Lanes 3, 4, 7, and 8). Cells were harvested, and the levels of apoptosis were determined by TUNEL assay.

tion assay. The NFκB inhibitor parthenolide inhibited NFκB activity in a dose-dependent manner (Fig. 7A, Lanes 4 and 6). As a negative control, we also used a transfected mutant form of IκB-α (mIκB-α), which cannot be phosphorylated by IKK, to inhibit NFκB activity, as shown in Fig. 7A, Lane 8. To show the effect of NFκB on inhibition of JNK activity, LNCaP cells were transfected with pCMX-mIκB-α and treated with 1 nM TPA for 24 h (Fig. 7B, Lanes 2, 4, and 6). Western blot analyses were performed using an antibody for phosphorylated JNK. Levels of phosphorylated JNK were increased in cells transfected with mIκB-α (Fig. 7B, Lane 2) or in cells treated with

parthenolide (Fig. 7B, Lane 4). These data suggest that an increase in JNK activity is linked to suppression of NFκB.

Suppression of JNK activity by NFκB seems inconsistent with data showing that treatment of LNCaP cells with TPA for 24 h results in the induction of both NFκB (Fig. 4, A and B) and JNK activity (Fig. 6). To clarify the role of NFκB in regulating JNK activity, we determined the activity of JNK over 72 h after treatment with TPA. LNCaP cells were treated with TPA, and the level of phosphorylated c-jun (a JNK substrate) was determined at 24, 48, and 72 h. By 48 h, the level of c-jun phosphorylation declined to basal levels, as shown in Fig. 7C. In another experiment, we determined the levels of activated c-JNK both in the presence and absence of an inhibitor of NFκB. As shown in Fig. 7D, Lane 2, the activity of JNK was elevated at 24 h but had declined to basal levels at 48 h. In the presence of the NFκB inhibitor parthenolide, JNK activity remained elevated at 48 h, showing that the decline in JNK activity is associated with the activity of NFκB.

Overall, our study presents data indicating that androgen/AR facili-

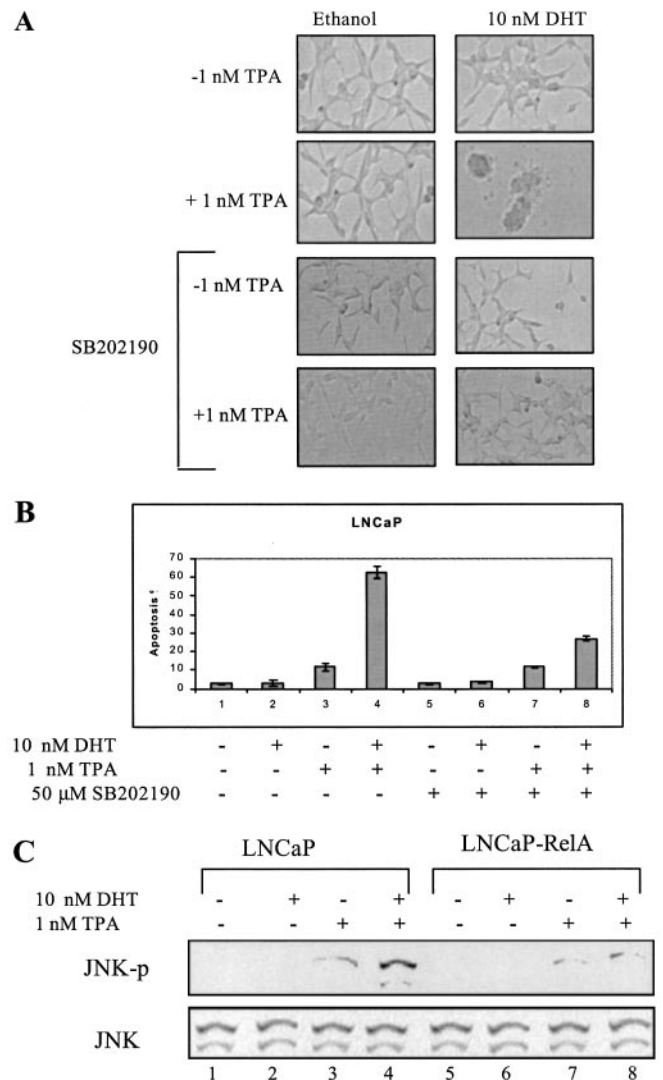


Fig. 6. Apoptosis signaling on combined treatment with 10 nM DHT and 1 nM TPA is mediated by JNK activity. A, LNCaP cells were treated with ethanol, 10 nM DHT, or DHT/TPA in the presence of SB202190 and analyzed by light microscopy or TUNEL assay (B). C, expression levels of JNK-P (active form of JNK) and total JNK protein were analyzed by Western blot with monoclonal antibodies against JNK or JNK-P. LNCaP and LNCaP-RelA cells were treated with ethanol (Lanes 1, 3, 5, and 7) or 10 nM DHT (Lanes 2, 4, 6, and 8) for 24 h, followed by treatment with 1 nM TPA for 24 h (Lanes 3, 4, 7, and 8). Equal amounts of lysate were resolved by 10% SDS-PAGE.

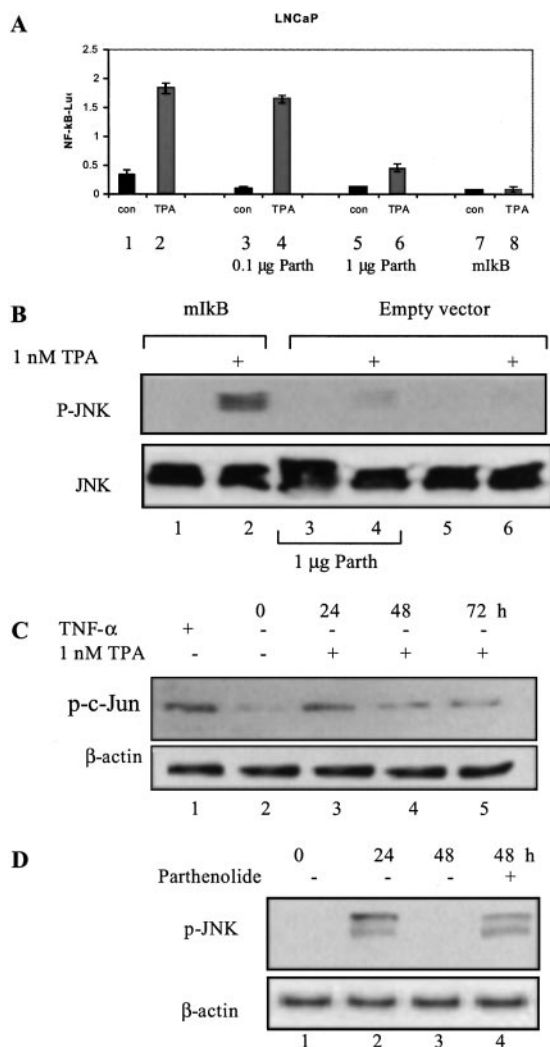


Fig. 7. Activation of JNK activity is linked to suppression of NFκB activity. *A*, the effect of NFκB inhibitors on NFκB-Luc activity in a transactivation assay. The NFκB inhibitor parthenolide inhibited NFκB activity in a dose-dependent manner, as shown in *Lanes 4* and *6*. We also used a transfected mutant form of IκB-α (mIkB-α) to inhibit NFκB activity, as shown in *Lane 8*. *B*, to demonstrate the effect of NFκB inhibition on JNK activity, LNCaP cells were transfected with pCMV-mIkB-α and treated with 1 nM TPA for 24 h (*Lanes 2, 4, and 6*). Western blot analysis was performed using an antibody to phosphorylated JNK. *C*, LNCaP cells were treated with TPA, and the level of phosphorylated c-Jun (a JNK substrate) was determined at 24, 48, and 72 h. TNF-α was used as a positive control for induction of c-Jun activity (*Lane 1*). *D*, the levels of activated c-JNK both in the presence and absence of an inhibitor of NFκB were determined. As shown in *Lane 2*, at 24 h the activity of JNK was elevated but at 48 h had declined to basal levels (*Lane 3*). In the presence of the NFκB inhibitor parthenolide, JNK activity remained elevated at 48 h (*Lane 4*).

ities TPA-induced apoptosis by interruption of NFκB signaling, which leads to activation of JNK and subsequent induction of caspase-3-dependent signaling. Fig. 8 summarizes the TPA/JNK/caspase-3 signaling pathway that leads to apoptosis in androgen-sensitive LNCaP prostate cancer cells.

DISCUSSION

TPA has been used as a tool for studying signal transduction pathways involved in cell proliferation (10) and apoptosis (9). TPA has also been used in low doses in a clinical trial to increase WBC counts in patients treated with cytotoxic drugs and has been shown to be beneficial in the treatment of myelocytic leukemia in patients refractory to standard antileukemic treatments (38). TPA has been reported to sensitize LNCaP cells to the apoptosis-inducing effect of

radiation (8, 9, 33). The sensitivity of LNCaP cells to TPA-induced apoptosis led to the hypothesis that androgen/AR could play a role in the signaling pathway, leading to apoptosis. In our study, we show that DHT sensitizes cells to TPA-induced apoptosis in the androgen-sensitive cell line LNCaP. This evidence suggests that androgen/AR influences TPA signaling either through changes in androgen-dependent gene expression or by direct interaction of the AR with signaling proteins affected by TPA.

A potential AR target is the inducible transcription factor NFκB (11). It has been shown that the activity of NFκB is elevated in androgen-independent prostate cancer cell lines PC-3 and DU145 compared with the androgen-sensitive prostate cancer LNCaP cell line (31, 32), suggesting that AR may suppress NFκB activity. AR-mediated suppression of NFκB could occur through several mechanisms. Cross-modulation, transcriptional interference, and physical interaction between AR and NFκB have been shown (20, 21). A direct interaction between AR and NFκB/RelA (data not shown) (21) might prevent RelA (p65) from binding to NFκB binding sites or affect the ability of RelA to translocate to the nucleus after activating NFκB. Increased expression of IκB induced by DHT (Fig. 4C) could be caused by the stabilization of IκBα protein or increased mRNA levels. Furthermore, pretreatment with 10 nM DHT, followed by 1 nM TPA, abrogated the TPA-mediated suppression of IκB (Fig. 4C, *Lane 4 versus Lane 3*), thus correlating with the ability of DHT to sensitize the cell to the proapoptotic influence of 1 nM TPA.

We have shown that the combination of TPA/DHT was unable to induce apoptosis in the LNCaP cell line overexpressing Rel A (LNCaP-Rel A; Fig. 5C). NFκB normally protects cells from induction of apoptosis by inhibition of signaling through the JNK pathway (9). We show that the combination of DHT/TPA activates the JNK pathway and that the normal protective effect of NFκB is suppressed by the actions of androgen/AR in LNCaP cells. In contrast, JNK activity was abrogated in the LNCaP-RelA cells (Fig. 6C).

Our data show the role of androgen/AR in modulating the induction of apoptosis in LNCaP cells, using low doses of TPA and pretreatment with DHT. This suggests that combining DHT and TPA might result in a more effective proapoptotic response than treatment with androgen alone, particularly in the context of intermittent androgen therapy. The effect of androgen in facilitating low-dose TPA induction of apoptosis was inhibited by the antiandrogen bicalutamide (Casodex, 1

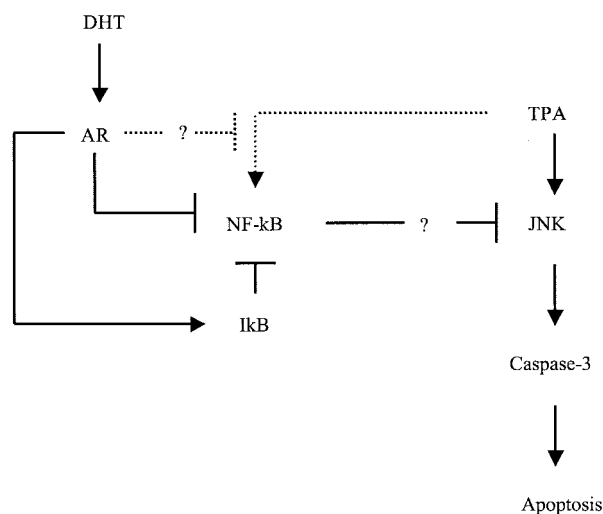


Fig. 8. Schematic illustration of the negative regulation of NFκB by androgen/AR by either enhancing expression of IκB or inhibiting signaling upstream of NFκB. Suppression of the negative effect of NFκB on JNK promotes TPA-induced apoptosis. The mechanism of controlling NFκB suppression of JNK is unknown.

μ M), a JNK inhibitor, and by overexpression of NF- κ B. Taken together, our results indicate that in androgen-sensitive prostate cancer cells such as LNCaP, androgen/AR facilitates TPA-induced apoptosis by interruption of NF- κ B signaling, which allows JNK activation to initiate the apoptotic caspase cascade through caspase-3. A key finding of our study is that androgen enables low concentrations of TPA (1 nM) to induce apoptosis.

Therapeutic approaches to selectively induce apoptosis by manipulating apoptotic pathways are being explored in a variety of clinical circumstances, and such approaches have been suggested as possible strategies to enhance intermittent androgen therapy in the treatment of prostate cancer (3, 6). The apoptotic process is subject to input from many different cell-signaling pathways. Understanding these pathways and their interactions is critical for effective integration of accumulating apoptosis-related information into therapeutic strategies. Here, we show that pretreatment with androgen facilitates induction of apoptosis with low concentrations of TPA. It may be possible that the effects of intermittent androgen therapy could be improved by agents that augment apoptosis (3, 5, 6, 39, 40). The observations described here suggest a mechanism whereby androgen influences the apoptotic propensity of tumor cells.

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