Suppression of Constitutive and Tumor Necrosis Factor α-Induced Nuclear Factor (NF)-κB Activation and Induction of Apoptosis by Apigenin in Human Prostate Carcinoma PC-3 Cells: Correlation with Down-Regulation of NF-κB-Responsive Genes

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ABSTRACT

Purpose: Development of androgen independence and resistance to apoptosis in prostate cancer are often correlated with high levels of serum tumor necrosis factor (TNF)-α in these patients. The loss of sensitivity to TNF-α-induced apoptosis in androgen-insensitive prostate carcinoma cells is due in part to constitutive activation of Rel/nuclear factor (NF)-κB transcription factors that regulate several cell survival and antiapoptotic genes. Our previous studies have demonstrated growth inhibitory and apoptotic effects of apigenin, a common plant flavonoid, in a variety of human prostate carcinoma cells. Here we examined whether apigenin is effective in inhibiting constitutive and tumor necrosis factor (TNF)-α-induced NF-κB activity.

Experimental Design: Using androgen-insensitive human prostate carcinoma PC-3 cells, the effect of apigenin was assessed on NF-κB activation by electrophoretic mobility shift assay and reporter gene assay. Expression of NF-κB subunits p65 and p50, IkBα, p-IκBα, in-beads kinase assay and NF-κB-regulated genes were determined by Western blot analysis. Apoptosis was determined by annexin V/propropidium iodide staining after fluorescence-activated cell-sorting analysis.

Results: Treatment of cells with 10–40 μM doses of apigenin inhibited DNA binding and reduced nuclear levels of the p65 and p50 subunits of NF-κB. Apigenin inhibited IκBα degradation and IκBα phosphorylation and significantly decreased IKKα kinase activity. Apigenin also inhibited TNF-α-induced activation of NF-κB via the IκBα pathway, thereby sensitizing the cells to TNF-α-induced apoptosis. The inhibition of NF-κB activation correlated with a decreased expression of NF-κB-dependent reporter gene and suppressed expression of NF-κB-regulated genes [specifically, Bcl2, cyclin D1, cyclooxygenase-2, matrix metalloproteinase 9, nitric oxide synthase-2 (NOS-2), and vascular endothelial growth factor].

Conclusions: Our results indicate that inhibition of NF-κB by apigenin may lead to prostate cancer suppression by transcriptional repression of NF-κB-responsive genes as well as selective sensitization of prostate carcinoma cells to TNF-α-induced apoptosis.

INTRODUCTION

The progression of prostate cancer from a localized and androgen-sensitive neoplasm to an invasive, metastatic, and deadly malignancy is commonly associated with loss of androgen dependence (1–3). The loss of androgen dependence is often correlated with overexpression of several antiapoptotic and cell survival genes that makes the cancer cells resistant to apoptosis (4, 5). Members of the Rel/nuclear factor (NF)-κB family control expression of a multitude of critical genes that regulate cell survival, proliferation, apoptosis, immune responses, and adaptive responses to changes in cellular redox balance (6–10). NF-κB consists of homodimers and heterodimers formed by several subunits: NF-κB1 (p50/p105); NF-κB2 (p52/100); RelA (p65); RelB; and c-Rel proteins (9, 10). The NF-κB proteins are regulated by inhibitors of the IκB family that includes IκBα, IκBβ, IκBε, IκBγ, Bcl-3, p100, and p105 (9, 10). In an inactive state, NF-κB is present in the cytoplasm as a heterodimer composed of p65, p50, and IκBα subunits. In response to various stimuli, the IκBα subunit is phosphorylated by an upstream IKKα at serine residues 32 and 36, triggering ubiquitination and proteasomal degradation of IκBα, thereby facilitating the translocation of p50-p65 heterodimer into the nucleus (11). Phosphorylation of p65 facilitates its binding to a specific sequence in DNA, which in turn results in gene transcription.

In recent years, the importance of NF-κB in the development and progression of cancer has been well recognized (12, 13). Inappropriate activation of NF-κB leads to upregulation of genes encoding adhesion molecules, inflammatory cytokines [tumor necrosis factor (TNF)-α], chemokines, growth factors, and antiapoptotic genes (13, 14). NF-κB activation has been implicated in the pathogenesis of many types of human cancers including hematological malignancies and cancer of the breast, colon, skin, lung, esophagus,
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uterine cervix, and prostate (15–23). Recent studies have reported that NF-κB is constitutively activated in human prostate cancer tissue, androgen-insensitive human prostate carcinoma cells, and prostate cancer xenografts (24–27). The signal transduction pathways that result in NF-κB activation involve tyrosine kinases, NF-κB-inducing kinase, and IKK; these kinases induce phosphorylation and faster turn-over of IκBα, the super-repressor of NF-κB activation (26, 27). Studies have suggested that increased NF-κB activity in androgen-insensitive human prostate carcinoma cells most likely makes them resistant to apoptosis and TNF-α-based chemotherapy (28–30). Therefore, agents capable of suppressing NF-κB activation may be potentially useful in the prevention and management of prostate cancer.

Studies from our laboratory and elsewhere (31–34) have demonstrated that apigenin (4’,5,7-trihydroxyflavone), a common dietary flavonoid abundantly present in fruits and vegetables, may prove useful in the prevention and therapy of prostate cancer. Similarly, other studies have shown that apigenin suppresses tumorigenesis and angiogenesis in highly malignant melanoma, breast, skin, and colon carcinoma cells (35–39). Many of these effects of apigenin are mediated through suppression of the expression of cyclooxygenase (COX)-2, matrix metalloproteinase (MMP)-9, nitric oxide synthase-2 (NOS-2), and lipoxygenase, which are regulated by NF-κB (40, 41). Apigenin has been shown to induce apoptosis in a wide variety of malignant cells including prostate cancer (31, 32), breast cancer (37), melanoma (35), colon cancer (39), leukemia (42), and thyroid cancer cells (43) through activation of caspases, inhibition of protein kinases, topoisomerase inhibition, and modulation of Bax and Bcl2 expression. Apigenin has been shown to inhibit activation of mitogen-activated protein kinase/ extracellular signal-regulated kinase 1/2, the upstream kinase cascades involved in NF-κB activation (44). Because NF-κB is constitutively activated in androgen-insensitive human prostate carcinoma PC-3 cells, and it is known that these cells are highly resistant to TNF-α-induced apoptosis, we elected to investigate the effects of apigenin on various steps in the TNF-α-induced NF-κB activation pathway. The results presented here demonstrate that apigenin inhibits both constitutive and TNF-α-induced NF-κB activation and that apigenin sensitizes PC-3 cells to TNF-α-induced apoptosis.

MATERIALS AND METHODS

Cell Lines and Reagents. Androgen-insensitive human prostate carcinoma PC-3 cells were obtained from American Type Culture Collection (Manassas, VA). RPMI 1640 and all other cell culture materials were obtained from Life Technologies, Inc. (Gaithersburg, MD). Apigenin (>95% purity) was obtained from A. G. Scientific, Inc. (San Diego, CA). NF-κB-dependent reporter plasmid (PathDetect NF-κB cis-Reporter System) with p-NF-κB-Luc plasmid and pFC-MEKK positive control plasmid was obtained from Stratagene, (La Jolla, CA). The Luciferase Assay System and β-Glo Assay System were purchased from Promega (Madison, WI). N-Acetyl leucyl leucyl norleucinal (ALLN) was purchased from Sigma Chemical Co. (St. Louis, MO). Human recombinant TNF-α was purchased from Roche Applied Sciences (Indianapolis, IN). Anti-NF-κB/p65, anti-NF-κB/p50, anti-IκBα, anti-NOS-2, anti-COX-2, anti-Bcl2 antibodies, IκBα-glutathione S-transferase substrate, and agarose-conjugated IKKα antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies for anti-MMP-9, anti-vascular endothelial growth factor (VEGF), and anti-cyclin D1 were obtained from Neomarkers (Fremont, CA). Phospho-IκBα antibody was purchased from Cell Signaling Technology (Fremont, CA). The ApopNexin FITC apoptosis detection kit containing FITC-labeled annexin V and propidium iodide was obtained from Intergen (New York, NY).

Cell Culture. The cells were cultured under standard culture condition in RPMI 1640 supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. The cells were maintained at 37°C and 5% CO2 in a humid environment. At 60% confluence, cultures were switched to media containing 1% fetal bovine serum for 12 h and then treated with specified doses of apigenin in DMSO or DMSO alone [maximum final concentration, 0.1% (v/v)], with or without TNF-α or ALLN for the indicated times. After the desired treatments, medium was aspirated, cells were harvested by the addition of trypsin-EDTA, and cytosolic and nuclear extracts were prepared as described previously (32).

Protein Extraction and Western Blot Analysis. After treatment of the cells as described above, media were aspirated, cells were washed with cold PBS (pH 7.4), and ice-cold lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na2VO4, 0.5% NP40, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (pH 7.4)] with freshly added protease inhibitor mixture (Protease Inhibitor Cocktail Set III; Calbiochem, La Jolla, CA) was added, and incubated over ice for 30 min. The cells were scraped, and lysate was collected in a microfuge tube and passed through a 21.5-gauge needle to break up cell aggregates. The lysate was cleared by centrifugation at 14,000 × g for 15 min at 4°C, and supernatant (total cell lysate) was used or immediately stored at −80°C. The protein concentration was determined by DC Bio-Rad assay using the manufacturer’s protocol (Bio-Rad Laboratories, Hercules, CA).

For Western blot analysis, an appropriate amount of cell lysates (25–50 μg protein) was resolved over 4–20% Tris-glycine polyacrylamide gel and then transferred onto the nitrocellulose membrane. The blots were blocked using 5% nonfat dry milk and probed using appropriate primary antibodies in blocking buffer overnight at 4°C. The membrane was then incubated with appropriate secondary antibody conjugated with horseradish peroxidase (Amersham Life Sciences Inc., Arlington Heights, IL) followed by detection using an enhanced chemiluminescence kit (ECL; Amersham Life Sciences Inc.). To ensure equal protein loading, the membrane was stripped and reprobed with anti-Oct-1 and anti-α-tubulin antibodies (Santa Cruz Biotechnology).

**Electrophoretic Mobility Shift Assay (EMSA) and Supershift Assay.** EMISA for NF-κB was performed using the Lightshift Chemiluminescent EMISA kit (Pierce, Rockford, IL) following the manufacturer’s protocol. Briefly, DNA was biotin labeled using the biotin 3‘-end-labeling kit (Pierce) in a 50-μl reaction buffer and 5 pmol of double-stranded NF-κB oligonucleotide (5‘-AGTTGAGGGGACTTTCCAGGC-3‘ and 3‘-TCAACTCTCCGAAAGGTCCCG-5‘) incubated in a microfuge tube with 10 μl of 5× terminal deoxynucleotidyltransferase buffer, 5 μl of 5 μM biotin-N4-CTP, 10 units of diluted terminal deoxynucleotidyltransferase, and 25 μl of ultrapure water at 37°C for 30 min. The reaction was stopped with 2.5 μl of 0.2 M EDTA. To extract labeled DNA, 50 μl of chloroform:isoamyl alcohol (24:1) were added to each tube and centrifuged at 13,000 × g. The top aqueous phase containing the labeled DNA was further used for binding reactions. Each binding reaction contained 1× binding buffer [100 mM Tris, 500 mM KCl, and 10 mM DTT (pH 7.5)], 2.5% glycerol, 5 mM MgCl2, 50 ng/μl poly(deoxyinosinic-deoxyctydilic acid), 0.05% NP40, 2.5 μg of nuclear extract, and 20–50 femtomoles of biotin-end-labeled target DNA. The contents were incubated at room temperature for 20 min. To this reaction mixture, 5 μl of 5× loading buffer were added, subjected to electrophoresis on a native polyacrylamide gel, and transferred to a nylon membrane. After transfer was completed, DNA was cross-linked to the membrane at 120 mJ/cm2 using a UV cross-linker equipped with a UV cross-linker purchasing from Silk Scientific Corp. (Orem, UT). The significance between the control and treated groups was assessed by Student’s t test, and P < 0.05 were taken as significant in the experiments.

**RESULTS**

**Apigenin Inhibits Constitutive NF-κB Activation and Nuclear Translocation of NF-κB/p65 and NF-κB/p50 Subunits in PC-3 Cells.** To examine the effect of apigenin on NF-κB constitutive activation, PC-3 cells were treated with various concentrations (10, 20, and 40 μM) of apigenin for 24 h. As shown in Fig. 1A, apigenin treatment of PC-3 cells inhibited constitutive NF-κB/p65 and NF-κB/p50 activation in a dose-dependent fashion. The level of NF-κB/p65 inhibition at 10, 20, and 40 μM doses of apigenin was found to be 20%, 50%, and 70% after 24 h of treatment. The inhibition in the levels of NF-κB/p50 was of a lower magnitude after apigenin treatment. A similar inhibitory effect of apigenin on constitutive NF-κB activation in PC-3 cells was observed in a time-dependent study (Fig. 1B). The level of NF-κB/p65 inhibition after treatment with 20 μM apigenin for 12, 24, and 48 h was found to be 20%, 50%, and 60%, respectively. Similarly, a time-dependent inhibition in the level of NF-κB/p50 was observed after apigenin treatment. In these studies analyzing the specificity of the NF-κB band, the addition of unlabeled NF-κB probe resulted in a decrease or disappearance of the bands (data not shown). To further confirm the specificity of NF-κB DNA binding, we performed a supershift assay with antibodies specific for NF-κB/p65 and NF-κB/p50. As shown in Fig. 1A, a strong supershift in the case of anti-p65, but a weak shift for anti-p50 to a higher molecular weight band, suggesting that the observed NF-κB band consisted of these two subunits.

**Next we determined the effect of apigenin on constitutive NF-κB activation in PC-3 cells. Immunoblot analysis of nuclear extracts from apigenin-treated samples revealed that levels of NF-κB/p65 and NF-κB/p50 subunits were decreased in a dose-and time-dependent manner (Fig. 2, A and B). Compared with vehicle control, a 30%, 50%, and 70% inhibition in NF-κB/p65 protein expression in the nucleus was observed at 10–20–, and 40–40 μM doses of apigenin, whereas 20%, 50%, and 60% inhibition was observed at 20 μM apigenin for 12, 24, and 48 h.
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To further confirm whether the observed inhibitory effect of apigenin on NF-κB is from blocking IkBα phosphorylation, we used ALLN, a proteasomal inhibitor that prevents the degradation of IkBα without having any effect on its phosphorylation. PC-3 cells were treated with apigenin (20 μM) for 16 h followed by ALLN (100 μg/ml) for 1 h, and the cytoplasmic extracts were analyzed for total and phosphorylated IkBα protein expression. As shown in Fig. 3C, a significant increase in the level of total IkBα was observed on treatment with either ALLN (130%) or apigenin (140%) or a combination of the two (170%). With regard to pIkBα levels in this experiment, ALLN treatment resulted in a 180% increase in pIkBα level; however, pretreatment with apigenin resulted in hyperphosphorylation of the IkBα level to 120% (Fig. 3D). These results confirm that the inhibitory effect of apigenin on constitutive NF-κB activation in PC-3 cells is mediated specifically by blocking the phosphorylation of IkBα. Similarly, treatment with apigenin (20 μM) for a shorter duration (6 and 12 h) resulted in similar changes in the levels of IkBα and pIkBα under identical experimental conditions (data not shown).

Apigenin Inhibits Phosphorylation of IkBα via Its Inhibitory Effect on IKKα Kinase Activity. Because phosphorylation of IkBα is mediated by IKKs, we next determined whether the inhibitory effect of apigenin on IkBα phosphorylation is mediated via IKKα kinase activity. As shown in Fig. 4A, treatment of PC-3 cells with 10-, 20-, and 40- μM doses of apigenin for 24 h resulted in a significant decrease in IKKα-associated kinase activity in a dose-dependent fashion. Compared with vehicle-treated control, the level of inhibition was 30%, 40%, and 50%, respectively. Similar effects were observed on treatment of cells with 20 μM apigenin (30%, 50%, and 70% decrease was observed at 12, 24, and 48 h, respectively; Fig. 4A). It is important to emphasize here that the degree of decrease observed in kinase activity by apigenin was comparable with the decrease observed in phosphorylation of IkBα. However, treatment of cells with apigenin did not result in any appreciable change in the level of total IKKα in the cytoplasmic extract (Fig. 4B). Furthermore, in an attempt to assess whether the inhibitory effect of apigenin on IKKα activity is a direct response or is mediated via an upstream event, we next performed an in vitro IKKα kinase activity assay in the presence of apigenin. For this assay, an equal amount of cytosolic protein from the control sample was immunoprecipitated with agarose-conjugated IKKα antibody; the immunocomplex was then incubated with 10, 20, and 40 μM apigenin plus the substrate for 30 min; and the kinase assay was then performed as detailed in “Materials and Methods.” Interestingly, the in vitro addition of the above-mentioned doses of apigenin to assay incubations resulted in 30%, 50%, and 80% inhibition of IKKα kinase activity (Fig. 4C). This observation suggests that the inhibitory effect of apigenin on IKKα kinase activity is rather a direct response and may not necessarily require an upstream event.

Apigenin Inhibits TNF-α-Induced NF-κB Activation via the IkBα Pathway and Sensitizes PC-3 Cells to TNF-α-Induced Apoptosis. To assess the effect of apigenin on TNF-α-induced activation of NF-κB, serum-starved cells were treated with 20 μM apigenin for 16 h either before or after TNF-α (10 ng/ml) treatment for 30 min. Vehicle-treated control and cultures treated with TNF-α alone were used as controls. Cytoplas-
mic and nuclear extracts were prepared from these cultures, and nuclear extracts were analyzed by EMSA for NF-kB DNA binding activity. Compared with vehicle-treated control, treatment of cells with TNF-α resulted in a 180% increase in NF-kB/p65 and a 120% increase in NF-kB/p50 DNA binding activity, respectively. Pre- and posttreatment of cells with apigenin significantly decreased the levels in both NF-kB/p65 and NF-kB/p50, suggesting that apigenin inhibits TNF-α-induced NF-kB activation and that pretreatment or posttreatment apigenin treatments have a comparable inhibitory response (Fig. 5A). Further investigation on cytoplasmic extracts from this experiment for total IκBα and pIκBα revealed that treatment with TNF-α resulted in a decrease (75%) in total IκBα, which was partially restored only in the case of posttreatment with apigenin to 50% (Fig. 5B). However, TNF-α-induced pIκBα levels (360% compared with control) were strongly inhibited by both pretreatment and posttreatment with apigenin to 38% and 45%, respectively (Fig. 5C).

Because TNF-α-induced NF-kB activity is the major factor in PC-3 cells that makes them resistant to apoptosis, we tested whether apigenin is effective in sensitizing these cells to TNF-α-induced apoptosis, possibly by virtue of its ability to inhibit NF-kB activity. As observed by annexin V/propidium iodide staining, TNF-α-induced apoptosis and that pretreatment or posttreatment with apigenin (20 and 40 μM) for 16 h or treated with TNF-α (10 ng/ml) for 30 min with or without pretreatment or posttreatment with apigenin (20 and 40 μM) for 16 h. Cell lysate was prepared for luciferase reporter and p-NF-kB-Luc plasmid luciferase reporter construct. The cells were treated with either DMSO or apigenin (20 and 40 μM) for 16 h or treated with TNF-α (10 ng/ml) for 30 min with or without pretreatment or posttreatment with apigenin (20 and 40 μM) for 16 h. Cell lysate was prepared for luciferase reporter

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**Fig. 2** Effect of apigenin on nuclear and cytoplasmic levels of nuclear factor (NF)-κB/p65 and NF-κB/p50 in human prostate carcinoma PC-3 cells. Western blot analysis of (A) NF-κB/p65 and (B) NF-κB/p50 in nuclear extracts and (D) NF-κB/p65 and (E) NF-κB/p50 in cytoplasmic extracts is shown. The cells were grown to 60% confluence, switched to 1% fetal bovine serum for 12 h, and then treated with either DMSO or apigenin (final concentration, 10, 20, or 40 μM) in DMSO at the indicated dose and times. Equal amounts of nuclear and cytosolic protein were resolved on 4–20% SDS-PAGE, transferred onto a nitrocellulose membrane, probed with appropriate primary and secondary antibodies, and visualized by enhanced chemiluminescence. To ensure equal protein loading, the membrane was stripped and re-probed with anti-Oct-1 and anti-α-tubulin (F) antibodies. The details are described in “Materials and Methods.” Quantification of bands was done by densitometric analysis and is shown as fold change as compared with vehicle control at the bottom of the bands.
Apigenin Suppresses NF-κB Activation in Prostate Cancer

Medical or surgical androgen ablation therapy is commonly used in the management of prostate cancers (45, 46). Most patients initially respond to this therapy, however, the disease frequently relapses with the outgrowth of androgen-independent and chemotherapy-resistant cancer (1–3). It has been observed that serum TNF-α levels in patients with relapsed androgen-resistant prostate cancers are often higher than those in untreated patients or patients in remission; elevated serum TNF-α levels correlate with poor prognosis (47). Studies have shown that androgen-insensitive prostate carcinoma cells (DU145 and PC-3) are resistant to TNF-α treatment (48). More recently, constitutive activation of NF-κB has been observed in these cell lines, a characteristic that may help these cells survive damage induced by proapoptotic stimuli (26, 27). Our earlier studies have shown that apigenin, a common dietary flavonoid abundantly present in fruits and vegetables, possesses remarkable growth inhibition properties and is selective in inducing apoptosis in a variety of prostate cancer cells without affecting gene assay. An almost 7-fold increase in luciferase activity over the vector control (pFC-MEKK plasmid) was observed after stimulation with TNF-α (Fig. 6A). With pretreatment of cells with apigenin, TNF-α-induced NF-κB-dependent luciferase activity was inhibited by 18% and 22% at 20 and 40 μM concentrations of apigenin. Furthermore, cells prechallenged with TNF-α and posttreated with apigenin exhibited a significant decrease in NF-κB-dependent luciferase activity by 45% and 76% at 20 and 40 μM concentrations of apigenin (Fig. 6A). These results demonstrate that apigenin inhibits NF-κB-dependent reporter gene expression induced by TNF-α.

Next we examined whether apigenin has the potential to inhibit induction of NF-κB-responsive genes by TNF-α. As shown in Fig. 6B, treatment of cells with TNF-α induced the expression of NF-κB-responsive genes, i.e., Bcl2, cyclin D1, COX-2, MMP-9, NOS-2, and VEGF, which was significantly inhibited by pretreatment and posttreatment incubation of cells with apigenin. These results further strengthen the role of apigenin in blocking TNF-α-induced NF-κB activation through transcriptional repression of NF-κB-responsive genes.

DISCUSSION

Medical or surgical androgen ablation therapy is commonly used in the management of prostate cancers (45, 46). Most patients initially respond to this therapy, however, the disease frequently relapses with the outgrowth of androgen-independent and chemotherapy-resistant cancer (1–3). It has been observed that serum TNF-α levels in patients with relapsed androgen-resistant prostate cancers are often higher than those in untreated patients or patients in remission; elevated serum TNF-α levels correlate with poor prognosis (47). Studies have shown that androgen-insensitive prostate carcinoma cells (DU145 and PC-3) are resistant to TNF-α treatment (48). More recently, constitutive activation of NF-κB has been observed in these cell lines, a characteristic that may help these cells survive damage induced by proapoptotic stimuli (26, 27). Our earlier studies have shown that apigenin, a common dietary flavonoid abundantly present in fruits and vegetables, possesses remarkable growth inhibition properties and is selective in inducing apoptosis in a variety of prostate cancer cells without affecting
normal cells (31). The present study demonstrates that apigenin strongly inhibits both constitutive and TNF-α-induced NF-κB activation along with transcriptional genes and sensitizes highly resistant PC-3 cells to TNF-α-induced apoptosis.

Several recent reports have shown that NF-κB plays a pivotal role in protection of cancer cells from apoptosis through activation of antiapoptotic genes (13, 14). Activation of NF-κB also renders TNF-α ineffective in inducing apoptotic death of cancer cells and may be the probable cause of a thriving toxic TNF-α environment in prostate cancer patients leading to NF-κB activation (48). It has been demonstrated that NF-κB is constitutively activated in androgen-insensitive prostate carcinoma cells because of aberrant activation of IKK, resulting in high levels of IκBα phosphorylation and turnover (26, 27). It is logical to devise strategies to manipulate the NF-κB pathway to induce androgen-resistant prostate carcinoma cells to undergo apoptosis. Previous strategies have focused on protein synthesis inhibitors, cyclophosphamide, and proteasomal inhibitor ALLN treatment that are highly cytotoxic to normal cells (49). Apigenin, in contrast, is a natural, nontoxic, nonmutagenic plant flavonoid proven to inhibit growth and induce apoptosis in both early- and advanced-stage prostate carcinoma cells (31–34). In the present study, we have demonstrated the potential of apigenin to inhibit DNA binding of NF-κB, IκBα degradation, IκBα phosphorylation, IKK activation, NF-κB/p65 nuclear translocation, and NF-κB-dependent reporter gene expression. The most significant finding of this study is that pretreatment of PC-3 cells with a combination of apigenin and TNF-α makes them more sensitive to apoptosis because the antiapoptotic signaling elicited by TNF-α-induced NF-κB activation is effectively blocked by apigenin. Our results provide convincing evidence that pretreatment and posttreatment of cells with apigenin resulted in significant inhibition of NF-κB activation and that this effect was mediated via the IκBα pathway. This could be one of the major mechanisms through which apigenin overcomes TNF-α insensitivity in human prostate carcinoma cells. A re-

Fig. 5 Effect of apigenin on tumor necrosis factor (TNF)-α-induced nuclear factor-κB activation and apoptosis in human prostate carcinoma PC-3 cells. The cells were grown to 60% confluence, switched to 1% fetal bovine serum for 12 h, and then treated either with DMSO for 16 h or with TNF-α (10 ng/ml) for 30 min with or without pretreatment or posttreatment apigenin (40 μM) for 16 h, and cytoplasmic and nuclear extracts were prepared. A, electrophoretic mobility shift assay for nuclear factor-κB in nuclear extract. Western blot analysis for IκBα (B) and pIκBα (C) in cytoplasmic extract. The details are described in “Materials and Methods.” In each case, quantitation of bands was done by densitometric analysis and is shown as fold change as compared with vehicle control at the bottom of the bands. D, quantitation of apoptosis. The cells were grown to 30–40% confluence, switched to 1% fetal bovine serum for 12 h, and then treated with either DMSO or apigenin (40 μM in vehicle) for 16 h or with TNF-α (10 ng/ml) for 30 min with or without pretreatment or posttreatment apigenin (40 μM) for 16 h. The cells were then harvested and processed for fluorescence-activated cell-sorting analysis of annexin V/propidium iodide-stained cells as described in “Materials and Methods.” The data are representative of three independent experiments with similar results. Api, apigenin. †, P < 0.001; ‡, P < 0.05 (Student’s t test, compared with control).
lated study suggests that blocking NF-κB activation by mutant IκBα is not sufficient to induce apoptosis or modify the response to cytotoxic drugs (50). Therefore, it is probable that along with IκBα inhibition, apigenin might interfere with other signaling events in the NF-κB pathway. Detailed studies are required to further evaluate these findings.

Accumulating studies have shown that NF-κB regulates a wide variety of genes whose products are involved in inflammation, carcinogenesis, and evasion of apoptosis (11–14). Constitutive activation of NF-κB may up-regulate the expression of inflammatory cytokines, chemokines, cell adhesion molecules, and inflammatory gene products such as COX-2 and NOS-2 (13, 14). Antiapoptotic genes that are regulated by NF-κB include genes encoding Bcl2-like proteins (Bcl2, Bcl-<sub>X</sub>L, and Nr13), inhibitors of apoptosis proteins (cIAP1 and cIAP2), and others such as PAR-4 (13, 51). Moreover, prometastatic genes such as interleukin 6, urokinase plasminogen activator, MMP-9, nitric oxide synthase-2, and vascular endothelial growth factor. The cells were grown to 60% confluence, switched to 1% fetal bovine serum for 12 h, and then treated either with DMSO for 16 h or with TNF-α (10 ng/ml) for 30 min without or with pretreatment or posttreatment apigenin (40 μM) for 16 h, and total cell lysates were prepared. Equal amounts of protein were resolved on 4–20% SDS-PAGE, transferred onto a nitrocellulose membrane, probed with appropriate primary and secondary antibodies, and visualized by enhanced chemiluminescence. The details are described in “Materials and Methods.” To ensure equal protein loading, the membrane was stripped and reprobed with anti-α-tubulin antibody. Quantitation of bands was done by densitometric analysis and is shown as fold change as compared with vehicle control at the bottom of the bands.

Fig. 6 Effect of apigenin on tumor necrosis factor (TNF)-α-induced nuclear factor (NF)-κB-dependent reporter gene and NF-κB-responsive genes in human prostate carcinoma PC-3 cells. The cells were grown to 30–40% confluence, switched to serum-free medium for 12 h, and transfected with NF-κB-Luc construct. Later, these cells were treated with either DMSO or apigenin (20 and 40 μM in vehicle) for 16 h or with TNF-α (10 ng/ml) for 30 min without or with pretreatment or posttreatment apigenin (20 and 40 μM) for 16 h. Cell lysate was prepared for the luciferase reporter gene assay. Luciferase activity normalized to β-galactosidase activity was determined in control and treatment groups as described in “Materials and Methods.” Results are expressed as fold activity over the activity of the vector control. Api, apigenin. B, Western blot analysis for NF-κB-responsive genes, i.e., Bcl2, cyclin D1, COX-2, MMP-9, nitric oxide synthase-2, and vascular endothelial growth factor. The cells were grown to 60% confluence, switched to 1% fetal bovine serum for 12 h, and then treated either with DMSO for 16 h or with TNF-α (10 ng/ml) for 30 min without or with pretreatment or posttreatment apigenin (40 μM) for 16 h, and total cell lysates were prepared. Equal amounts of protein were resolved on 4–20% SDS-PAGE, transferred onto a nitrocellulose membrane, probed with appropriate primary and secondary antibodies, and visualized by enhanced chemiluminescence. The details are described in “Materials and Methods.” To ensure equal protein loading, the membrane was stripped and reprobed with anti-α-tubulin antibody. Quantitation of bands was done by densitometric analysis and is shown as fold change as compared with vehicle control at the bottom of the bands.
suggesting that inhibition of NF-κB activation might inhibit prostate carcinogenesis. In the present study, we have demonstrated that pretreatment and posttreatment with apigenin suppressed TNF-α-induced expression of Bcl2, cyclin D1, COX-2, MMP-9, NOS-2, and VEGF. The inhibition of expression of these NF-κB-regulated genes may explain the antiproliferative effects of apigenin. Because of the role of the NF-κB pathway in prostate cancer cell survival, proliferation, and resistance to chemotherapies and radiation, our findings suggest that this signaling pathway represents a key molecular target for anticancer activity of apigenin. Inhibition of NF-κB activation has been shown to be an important mechanism of action of some natural polyphenols (53) and other plant–derived agents, such as green tea polyphenols (54), genistein (55), silibinin (56), resveratrol (57), and curcumin (58). It is tempting to speculate that combinations of these agents might prove useful in the prevention and therapy of prostate cancer. Mechanism-based studies with multiple agents that synergistically inhibit NF-κB activation in prostate cancer are needed to validate this possibility.

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