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Betulinic Acid Suppresses Constitutive and TNFα-induced NF-κB Activation and Induces Apoptosis in Human Prostate Carcinoma PC-3 Cells

Thangaiyan Rabi[1,2], **Sanjeev Shukla**[1,2], and **Sanjay Gupta**[1,2,3,*]

[1] Department of Urology, The James & Eillen Dicke Research Laboratory, Case Western Reserve University, Cleveland, Ohio 44106

[2] University Hospitals Case Medical Center, Cleveland, Ohio 44106

[3] Case Comprehensive Cancer Center, Cleveland, Ohio 44106

Abstract

Development of chemoresistance in androgen-refractory prostate cancer cells is partly due to constitutive activation of Rel/NF-κB transcription factors that regulate several cell survival and anti-apoptotic genes. In this study we examined whether betulinic acid (BetA), a pentacyclic triterpene from the bark of white birch, is effective in inhibiting NF-κB expression in androgen-refractory human prostate cancer cells exhibiting high constitutive NF-κB expression. Treatment of PC-3 cells with BetA inhibited DNA binding and reduced nuclear levels of the NF-κB/p65. BetA-mediated NF-κB inhibition involved decreased IKK activity and phosphorylation of IκBα at serine 32/36 followed by its degradation. Reporter assays revealed that NF-κB inhibition by BetA is transcriptionally active. These effects were found to correlate with a shift in Bax/Bcl-2 ratio and cleavage of poly(ADP)ribose polymerase more towards apoptosis. BetA also inhibited TNFα-induced activation of NF-κB *via* the IκBα pathway, thereby sensitizing the cells to TNFα-induced apoptosis. Our studies demonstrate that BetA effectively inhibits constitutive NF-κB activation and supports the rationale for targeting NF-κB through combination protocols with BetA in androgen-refractory prostate cancer.

Keywords

transcription factor; betulinic acid; IκBα; IKK; prostate cancer

INTRODUCTION

Nuclear Factor-*kappa*B (NF-κB), a member of *Rel* transcription factor family, controls expression of a multitude of critical genes that regulate cell survival, proliferation, apoptosis, immune responses and adaptive responses to changes in cellular redox balance [1–3]. NF-κB consists of homo- and hetero-dimers formed by several subunits: NF-κB1 (p50/p105), NF-κB2 (p52/100), RelA (p65), RelB, and c-Rel proteins [4,5]. The NF-κB proteins are regulated by inhibitors of the IκB family which includes IκBα, IκBβ, IκBε, IkBγ, Bcl-3, p100 and p105 [6]. In an inactive state, NF-κB is present in the cytoplasm as a heterodimer composed of p65, p50 and IκB 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

*Correspondence to: Sanjay Gupta, Ph.D., Department of Urology, The James & Eillen Dicke Research Laboratory, Case Western Reserve University, 10900 Euclid Avenue, Cleveland, OH 44106, Phone: (216) 368 6162, Fax: (216) 368 0213, sanjay.gupta@case.edu.

degradation of IκBα, thereby facilitating the translocation of p50–p65 heterodimer into the nucleus [4–6]. Phosphorylation of p65 facilitates its binding to a specific sequence in DNA, which in turn results in gene transcription [4]. In recent years, increasing evidence indicates that activation of NF-κB plays an important role in coordinating the control of apoptotic cell death, consistent with reports that NF-κB can promote apoptosis under certain circumstances [5,6]. Aberrant NF-κB activation has been implicated in the pathogenesis of several human malignancies, including various types of carcinoma, and hematological cancers including leukemia and lymphoma [7]. We and others have reported that NF-κB/p65/RelA is constitutively activated in human prostate cancer and prostate cancer xenografts [8–10]. Nuclear localization of NF-κB/p65 in primary prostate cancer is highly predictive of metastasis, disease relapse and resistance to chemotherapy [11,12]. NF-κB has been shown to activate a transcription regulatory element of the prostate-specific antigen-encoding gene, a marker of prostate cancer development and progression [13]. Increased NF-κB activity in androgen-refractory human prostate carcinoma PC-3 cells contributes directly to its aggressive behavior [14]. Conversely, blockade of NF-κB activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion and metastasis [15]. Therefore, agents that can suppress NF-κB activation are attractive candidates for development as therapeutic agents for the management of prostate cancer.

Betulinic acid (BetA) is a pentacyclic triterpene discovered in 1995 in the stem bark of the plant *Zizyphus mauritiana*; it was found to be a melanoma-specific cytotoxic agent that inhibits the growth of human melanoma tumors in athymic mice [16]. BetA is also found in various other plants widespread in tropical regions including *Tryphyllum peltaum*, *Ancistrocladus heyneaus*, *Zizyphus joazeiro*, *Diospyoros leucomelas*, *Tetracera boliviana*, and *Syzygium formosanum* [17]. BetA has been shown to induce apoptosis in neuroblastomas and glioblastomas through the mitochondrial activation pathway [18,19]. BetA also appears to be active against HIV [17,20], and it has displayed anti-inflammatory activities in various experimental systems [21]. Reports have demonstrated that apoptosis induced by BetA involved mitogen-activated protein kinase activation without involvement of caspases [22]. The cytotoxicity research on BetA showed that it had selective cytotoxicity on tumor cell lines but not on normal cells [23], suggesting that it may have potential for development as a therapeutic agent. Recent evidence indicates that the anticancer activity of BetA can be markedly increased when it is used in combination with chemotherapy, ionizing radiation or TRAIL [24–26]. BetA has been shown to suppress carcinogen-induced NF-κB activation through inhibition of IκBα kinase and p65 phosphorylation in epithelial cells and through inhibition of the transcription factors specificity protein1 (Sp1), Sp3, and Sp4 which regulate VEGF and survivin expression [27,28]. We investigated the effect of BetA on constitutive NF-κB inhibition and in the TNFα-induced NF-κB activation pathway. Our results demonstrate that BetA inhibits both constitutive and TNFα-induced NF-κB activation, and that BetA sensitizes PC-3 cells to TNFα-induced apoptosis.

MATERIALS AND METHODS

Cell Lines and Reagents

Androgen-refractory human prostate cancer PC-3 cells were obtained from ATCC (Manassas, VA). RPMI 1640 medium and all other cell culture materials were obtained from Life Technologies Inc., (Gaithersburg, MD). Betulinic acid (≥ 98% purity) was obtained from A. G. Scientific, Inc. (San Diego, CA). NF-κB-dependent reporter plasmid (PathDetect® NF-κB *cis*-Reporter System) having p-NF-κB-Luc plasmid and pFC-MEKK positive control plasmid was obtained from Stratagene, (La Jolla, CA). The Luciferase Assay System and Beta-Glo™ Assay System were purchased from Promega Corporation (Madison, WI). Proteasomal inhibitor MG132 was obtained from Calbiochem San Diego, CA. Human recombinant tumor

necrosis factor-α (TNFα) was purchased from Roche Applied Sciences (Indianapolis, IN). Anti-NF-κB/p65, anti-IκBα, anti-p-IκBα, anti-IKKα, anti-Bax, anti-Bcl-2 and anti-PARP antibodies were purchased from Santa Cruz Biotechnologies Inc. (Santa Cruz, CA). The Cell Death detection ELISA^{PLUS} kit was obtained from Roche (Mannheim, Germany) and the ApopNexin® FITC apoptosis detection kit containing FITC-labeled annexin V and PI was obtained from Millipore (Billerica, MA).

Cell Culture

The cells were cultured under standard culture condition in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The cells were maintained at 37°C and 5% CO_2 in a humid environment. At 60% confluency, cultures were switched to 1% FBS for 12 h, and then treated with specified doses of BetA in DMSO or DMSO alone (maximum final concentration 0.1%, v/v), or with/without TNFα, or with/without MG-132 for indicated times. After desired treatments, medium was aspirated and cells were harvested by the addition of Trypsin-EDTA, and cytosolic and nuclear extracts were prepared as previously described [29].

Protein Extraction and Western Blot Analysis

Following treatment of cells as described above, media was 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 Na_3VO_4, 0.5% NP-40, 1% Triton X-100, 1 mM PMSF (pH 7.4)] with freshly added protease inhibitor cocktail (Protease Inhibitor Cocktail Set III, Calbiochem, La Jolla, CA) was added and it was incubated over ice for 30 min. The cells were scraped, lysate was collected in a microfuge tube and passed through a 21½ G 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 manufacturer's protocol (Bio Rad Laboratories, Hercules, CA).

For Western blot analysis, appropriate amount of cell lysates (25–50 μg protein) were resolved over 4–20% Tris-Glycine polyacrylamide gel and then transferred onto the nitrocellulose membrane. The blots were blocked using 5% non-fat 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 (HRP) (Amersham Life Sciences Inc., Arlington Heights, IL) followed by detection using chemiluminescence ECL kit (Amersham Life Sciences Inc., Arlington Heights, IL). To ensure equal protein loading, the membrane was stripped and reprobed with anti-Oct-1 and anti-α-tubulin antibodies (Santa Cruz Biotechnologies).

Electrophoretic Mobility Shift Assay (EMSA)

EMSA for NF-κB was performed using Lightshift™ Chemiluminiscent EMSA kit (Pierce, Rockford, IL) following manufacturer's protocol. Briefly, DNA was biotin-labeled using the Biotin 3′ end labeling kit (Pierce, Rockford, IL) in a 50 μl reaction buffer, 5 pmol of double stranded NF-κB oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′ and 3′-TCAACTCCCCTGAAAGGGTCCG-5′) incubated in a microfuge tube with 10 μl of 5x TdT (terminal deoxynucleotidyl transferase) buffer, 5 μl of 5 μM biotin-N4-CTP, 10 U of diluted TdT, 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) was 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 1x-binding buffer (100 mM Tris, 500 mM KCl, 10 mM Dithiothretol, pH 7.5), 2.5% glycerol, 5 mM $MgCl_2$, 50 ng/μl poly (dI-dC), 0.05% NP-40, 2.5 μg of nuclear extract and 20–50 fm of biotin-end-labeled

target DNA. The contents were incubated at room temperature for 20 min. To this reaction mixture, 5 μl of 5x loading buffer was added, subjected to gel 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/cm^2 using a UV cross-linker equipped with 254-nm bulb. The biotin end-labeled DNA was detected using streptavidin-horseradish peroxidase conjugate and a chemiluminiscent substrate. The membrane was exposed to X-ray film (XAR-5 Amersham Life Science Inc., Arlington Height, IL) and developed using a Kodak film processor.

Cell Survival Assay

The effect of BetA on the viability of cells was determined by MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazoliumbromide) assay. Briefly, the cells were plated at 1×10^4 cells per well in 200 μl of complete culture medium containing desired concentrations of BetA prepared in DMSO and diluted with the culture media to achieve 1–40 μM final concentrations. The concentration of DMSO remained within maximum permissible concentration of 0.1% in both control and treated samples. After incubation for desired times at 37°C in a humidified incubator, cell viability was determined. 50 μl MTT (5 mg/ml in phosphate buffered saline stock, diluted to working strength 1mg/ml with media) was added to each well and incubated for 2 h after which the plate was centrifuged at X 600*g* for 5 min at 4°C. The MTT solution was removed from the wells by aspiration. After careful removal of the medium, 0.1 ml of buffered DMSO was added to each well, and plates were shaken. The absorbance was recorded on a microplate reader at the wavelength of 540 nm. The effect of BetA on growth inhibition was assessed as percent cell viability where vehicle-treated cells were taken as 100% viable.

Apoptosis Detection by ELISA

Apoptosis was assessed by Cell Death Detection ELISA^{PLUS} kit according to the manufacturer's protocol. Briefly, cells were treated with 0.1% DMSO or 5–20 μM BetA for 48 h, after which the media was aspirated, the cells were washed with cold PBS and incubated on ice for 30 min. in cell lysis buffer. The cells were then scraped and the lysate was collected in a microfuge tube and vortex to break up the cell aggregates. The lysate was cleared by centrifugation at 5,000 rpm for 10 min at 4°C and the supernatant was stored at −80°C. Protein concentration was determined by DC Bio-Rad protein assay. The cell lysate with 50 μg protein were added to lysis buffer provided with the kit and pipetted on a streptavidin-coated 96-well microtiter plate to which immunoreagent mix was added and incubated for 2 h at room temperature with continuous shaking at 500 rpm. The wells were then washed with washing buffer, and color was developed by addition of substrate solution, which was read at 405 nM against the blank, reference wavelength of 490 nm after 10–15 min. The enrichment factor (total amount of apoptosis) was calculated by dividing the absorbance of the sample ($A_{405\ nm}$) by the absorbance of the controls without treatment ($A_{490\ nm}$).

Transfection and Reporter Assay

To examine the effect of BetA on TNFα-induced reporter gene expression, PC-3 cells were transiently transfected using Lipofectamine™ 2000 reagent (Invitrogen Corporation, Carlsbad, CA) with 1.6 μg p-NF-κB-Luc plasmid for 6 h in serum free medium. Later these cells were switched to 5% FBS medium treated either with DMSO or BetA (20μM in vehicle) for 16 h, or with TNFα (10 ng/ml) for 30 min without or with pre-BetA treatment (20μM) for 16 h. The cells were processed to obtain total cell lysate for luciferase reporter gene assay and β-galactosidase assay (Promega, Madison, WI) according to vendor's protocol. Luciferase activity was normalized to β-galactosidase activity to control for transfection efficiency.

Detection of Apoptosis by FACS Analysis

PC-3 cells (30–40% confluent) grown in 6-well plates were switched to 1% FBS media for 12 h and then treated either with TNFα (10 ng/ml) only, or pretreated with BetA (20μM) only for 16 h and later with TNFα for 30 min. After these treatments, cells were harvested and processed for annexin V-PI staining using ApopNexin® FITC apoptosis detection kit as per manufacturer's protocol. The samples were then analyzed for apoptotic cells by FACS analysis.

Statistical Analysis

Densitometric measurements of the bands in Western blot analysis were performed using digitalized scientific software program on Kodak 2000R Image Station. Statistical analysis was performed using one-way analysis of variance (ANOVA) with 95% confidence limits. Data are presented in the figures as mean ± SE.

RESULTS

Since inappropriate regulation of NF-κB/p65/RelA correlates with prostate cancer progression, therefore, as a first step to determine the constitutive activation of NF-κB, we analyzed the expression of NF-κB/p65 and IκBα in various human prostate cancer cells. As shown in figure 1A, protein expression of NF-κB/p65 and IκBα was higher in androgen-refractory human prostate cancer DU145 and PC-3 cells compared to androgen-responsive LNCaP cells. Since NF-κB activation requires IκBα phosphorylation at Ser32/36 followed by ubiquitination and degradation, we determined the phosphorylated levels of IκBα in LNCaP, DU145 and PC-3 cells. IκBα is more highly phosphorylated in androgen-refractory DU145 and PC-3 cells than in androgen-responsive LNCaP cells. A modest decrease in the levels of IKKα was observed in DU145 and PC-3 cells compared to LNCaP cells (Figure 1A). Next we determined DNA binding for NF-κB/p65, which was higher in DU145 and PC-3 cells compared to LNCaP cells (Figure 1B). It is well recognized that degradation of phosphorylated IκB by the 26 S proteasome regulates the DNA binding of NF-κB subunits therefore; next we used MG132, a pharmacological inhibitor of proteasome activity, to determine the effect of treatment in these cells. Previous study has demonstrated that use of proteasomal inhibitor MG132 blocks degradation of phosphorylated IκBα [30]. Exposure of LNCaP, DU145 and PC-3 cells to 20 μM MG132 for 6 and 12 h decreased the rate of IκBα phosphorylation, similar to published observations (Figure 1C). Exposure of cells to MG132 for 12 h resulted in accumulation of phosphorylated IκBα protein in all three cell lines; however, the rate of p-IκBα accumulation was faster in PC-3 cells whereas the final amount of the p-IκBα protein was higher in DU145 and PC-3 cells compared to LNCaP cells, as evidenced by time-dependent studies (Figure 1C). MG132 treatment significantly inhibited the DNA binding activity of NF-κB/p65 at 12 h in all three prostate cancer cell lines (Figure 1D). Based on the rapid turnover of IκBα in PC-3 cells we considered this cell line for further studies with BetA.

Our next aim was to investigate whether BetA treatment imparts anti-proliferative effects against androgen-refractory human prostate carcinoma PC-3 cells. BetA is a naturally occurring pentacyclic triterpenoid which has shown to possess anti-viral, anti-inflammatory and anticancer properties (Figure 2A). Exposure of PC-3 cells to 20 μM concentration of BetA for 24 h resulted in loss of adhesion to substrate, and altered morphology, consistent with apoptosis (Figure 2B). Furthermore, BetA treatment (1 to 40 μM) resulted in a dose-dependent inhibition of cell growth, as compared to vehicle-treated controls. Exposure to BetA at the highest doses of 40 μM resulted in 91.2% to 96.7% decrease in cell viability between 24 to 72 h. BetA treatment for 24 h resulted in dose-dependent reduction in cell viability which ranged from 2.9% to 91.2% in PC-3 cells at 1 to 40 μM concentrations. BetA treatment also resulted in time-dependent inhibition of cell growth. This effect was more pronounced at 48 and 72 h post-treatment with BetA (Figure 2C). We next investigated whether BetA-mediated loss of

cell viability in human prostate cancer PC-3 cells is a result of apoptosis. To demonstrate these effects we performed cell death detection by ELISA. Compared to vehicle treated controls, 1.4, 2.5 and 4.8 fold increases in induction of apoptosis were observed with BetA in PC-3 cells, following 24 h of treatment (Figure 2D). Since BetA treatment to PC-3 cells resulted in decreased cell survival and induction of apoptosis therefore, next we studied its effect on NF-κB signaling.

To examine the effect of BetA on NF-κB constitutive activation, PC-3 cells were treated with 10 and 20 μM BetA for 12, 24 and 48 h. As shown in figure 3A, BetA treated cells showed exclusive cytoplasmic accumulation of NF-κB/p65 protein inhibiting its translocation into the nucleus in time and dose dependent manner. This effect correlated with significant dose and time dependent increase in the levels of total IκBα. To further determine whether inhibition of BetA on constitutive activation of NF-κB was caused by inhibition of IκBα degradation, we examined the cells for p-IκBα status in the cytoplasm. The level of serine 32-phosphorylated IκBα (p-IκBα) exhibited a dose and time dependent decrease in the phospho-protein expression after BetA treatment. Because phosphorylation of IκBα is mediated by IKKs, we next determined whether the inhibitory effect of BetA on IκBα phosphorylation is mediated *via* IKKα. BetA treatment markedly decreased the protein level of IKKα over 48 h. This finding raised the possibility that the primary target of BetA in the regulation of the IκB/NF-κB pathway might be regulated via IKKα. Furthermore, we examined the effect of BetA on DNA binding activities of NF-κB by EMSA assay in nuclear extracts of PC-3 cells. Treatment with 20 μM BetA for 24 h resulted in a decrease of NF-κB DNA binding (Figure 3B). Taken together, these experiments demonstrate that BetA inhibits constitutive activation of NF-κB in human prostate cancer PC-3 cells.

Since BetA prevents the entry of NF-κB/p65 into the nucleus, it is plausible that it might induce alterations in survival factors such as Bcl-2; therefore we next studied the time and dose dependent effects of BetA on the constitutive protein levels of Bax and Bcl-2 in PC-3 cells. The time-dependent treatment with 10 and 20 μM concentration of BetA resulted in the increase accumulation of Bax protein, which was observed up to 48 h post-treatment compared to the basal levels. In sharp contrast, the protein expression of Bcl-2 was significantly decreased by BetA treatment in a dose and time dependent fashion (Figure 4A). This resulted in a significant time and dose dependent shift in the ratio of Bax to Bcl-2 after BetA treatment which correlated with the cleavage of poly(ADP)ribose polymerase, indicative of induction of the apoptotic process (Figure 4B).

Since cytokine stimulation leads to NF-κB activation, we assessed the effect of BetA on TNFα-induced activation of NF-κB. PC-3 cells were serum starved and pretreated with 20 μM BetA for 16 h followed by TNFα (10 ng/ml) treatment for 30 min. Vehicle treated controls and cultures exposed only to TNFα were used as controls. Cytoplasmic and nuclear extracts were prepared from these cultures, and nuclear extracts were analyzed by EMSA for NF-κB DNA binding activity. Compared to vehicle treated control, treatment of cells with TNFα resulted in a significant increase in NF-κB/p65 DNA binding activity. Pre-treatment of cells with BetA significantly reduced NF-κB/p65 DNA binding activity, suggesting that BetA inhibits TNFα-induced NF-κB activation (Figure 5A). Further investigation on cytoplasmic extracts from this experiment, for total IκBα and p-IκBα, revealed that treatment with TNFα resulted in an increase in total IκBα, which was further increased after pretreatment with BetA. However, TNFα-induced p-IκBα levels were strongly inhibited by treatment with BetA (Figure 5B).

Although we have shown by EMSA that BetA blocks NF-κB activation, however, DNA binding does not always correlate with NF-κB-dependent gene transcription, suggesting there are additional regulatory steps. To determine the effect of BetA on TNFα-induced NF-κB-

dependent reporter gene expression, we transiently transfected the cells with the NF-κB-regulated reporter construct, incubated them with 20 μM BetA for 16 h, and then stimulated the cells with 10 ng/ml TNFα for 30 min. A 4.4-fold increase in NF-κB-Luc activity over the vector control was observed upon stimulation with TNFα, and BetA completely suppressed the TNFα-induced stimulation (Figure 5C). These results demonstrate that BetA also represses NF-κB-dependent reporter gene expression induced by TNFα.

Since TNFα-induced NF-κB activity is the major factor in PC-3 cells that make them resistant to apoptosis, we tested if BetA is effective in sensitizing these cells to TNFα-induced apoptosis, possibly by virtue of its ability to inhibit NF-κB activity. As observed by annexin V-PI staining, TNFα treatment of PC-3 cells resulted in apoptotic cell death rates that were comparable to those observed in vehicle treated controls; BetA exposure alone resulted in a 33% increase in rate of apoptotic cell death over controls. However, pre-treatment of cells with BetA followed by TNFα treatment resulted in a significant increase (44%; $p<0.001$) in the percentage of apoptotic cells (Figure 5D). These observations clearly suggest that BetA has the potential to sensitize highly resistant PC-3 cells to TNFα-induced apoptosis and that this effect of BetA may be mediated *via* inhibition of TNFα-induced NF-κB activation in these cells.

DISCUSSION

BetA is a natural product identified in various bark extracts and is readily synthesized from betulin, a major component in the bark of birch trees [17]. The initial report of the efficacy of BetA as a selective inhibitor of human melanoma cells was published in 1995 [16]. Subsequently, BetA was found to be active against neuroectodermal tumors (neuroblastoma, medulloblastoma, Ewing's sarcoma), malignant brain tumors, and several types of human epithelial malignancies, including carcinoma of the ovary, colon, breast, and lung, and squamous cell carcinoma of the head and neck [18,19,31–33]. Currently BetA is undergoing development as a therapeutic agent against melanoma with assistance from the Rapid Access to Intervention Development (RAID) program of the National Cancer Institute [34]. In this study we demonstrate that BetA causes growth inhibition and apoptosis in human prostate cancer cells.

It has been demonstrated that BetA induces apoptosis in several different cancer cell lines through multiple pathways, which include mitochondrial pathways, p53-independent induction of p21/Waf1, upregulation of death receptors, inhibition of Specificity Protein (Sp) transcription factors and interaction with other agents [16–28]. Studies have demonstrated that BetA-induced apoptosis in human prostate cancer LNCaP cells may be due to decreased AR expression, leading to decreased expression of VEGF and survivin and caspase-dependent PARP cleavage [28]. However, it is unclear how BetA causes apoptosis in androgen-refractory cell lines, which are highly resistant to pro-apoptotic stimuli. In this study we demonstrate that BetA induces apoptosis in androgen-refractory PC-3 human prostate cancer cells, and in addition sensitizes these cells to TNFα-induced apoptosis through suppression of NF-κB.

The transcription factor, NF-κB is a key mediator of the cellular stress response and in cells exposed to anticancer therapy NF-κB typically activates survival pathways [1–3]. Recent findings also suggest an inhibitory role of NF-κB in carcinogenesis and tumorigenesis [35]. It has been demonstrated that inhibition of NF-κB activity in cancer cell lines could reduces cell proliferation and metastatic capabilities *in vivo* [4–6,15]. We and others have shown that NF-κB/p65/RelA is constitutively activated during prostate cancer progression and in androgen-refractory human prostate cancer cells [8–10]. The major mechanism of NF-κB activation in prostate cancer cells involves aberrant activation of IKK, resulting in increased phosphorylation and instability of IκB proteins. It is logical to devise strategies to manipulate the NF-κB pathway in order to induce androgen-refractory prostate carcinoma cells to undergo

apoptosis. Previous strategies have employed protein synthesis inhibitors, cyclophosphamide, proteasomal inhibitor, ALLN and MG132 treatment, all of which are highly cytotoxic to normal cells [36,37]. BetA, in contrast, is a natural minimally-toxic, non-mutagenic pentacyclic triterpenoid proven to inhibit growth and induce apoptosis in both early and advanced stage cancers [38]. In the present study we have demonstrated the capability of BetA to inhibit DNA binding of NF-κB and its translocation to the nucleus, IκBα phosphorylation, and IKK activation. Our results provide convincing evidence that treatment of cells with BetA resulted in significant inhibition of NF-κB activation, and that this effect was mediated *via* the IκBα pathway. Our findings indicate that BetA shows potential for use as an agent targeting NF-κB blockage in androgen-refractory human prostate cancer cells. Although a related study suggests that blocking NF-κB activation by mutant IκBα is not sufficient to induce apoptosis or modify response to cytotoxic drugs [39]; it can be hypothesized that along with IκBα inhibition, BetA might interfere with other signaling events in the NF-κB pathway. Additional detailed studies are required to further evaluate these possibilities.

Studies have shown that androgen-refractory prostate cancer DU145 and PC-3 cells are resistant to TNFα treatment [40]. This resistance may be due to constitutive activation of NF-κB, a characteristic that has been observed in these cell lines and that may help these cells survive damage induced by pro-apoptotic stimuli [41]. Activation of NF-κB also renders TNFα ineffective in inducing apoptotic death of cancer cells and may cause the induction of a toxic TNFα environment in prostate cancer patients [40,41]. The most significant finding of this study is that pretreatment of PC-3 cells with a combination of BetA and TNFα make them more sensitive to apoptosis, since the anti-apoptotic signaling elicited by TNFα-induced NF-κB activation is effectively blocked by BetA. This could be one of the major mechanisms through which BetA might overcome TNFα insensitivity in human prostate cancer cells. Previous reports have shown that a combination of BetA and the cytotoxic ligand TRAIL significantly enhanced BetA-induced apoptosis [26]. Furthermore, BetA has been reported to act in concert with ionizing radiation or anticancer agents, suggesting that NF-κB inhibition might lead to such responses [24,25]. However, a recent study has demonstrated that BetA activates NF-κB in various tumor cell lines derived from neuroblastoma, glioblastoma and melanoma, unexpectedly promoting BetA-induced apoptosis [42]. A detailed study on BetA-induced NF-κB-mediated cell growth inhibitory responses in different cell cancer cell types is needed to resolve the issue of cell type-specific responses to BetA exposure. Because the NF-κB pathway plays such important roles in prostate cancer cell survival, proliferation and resistance to chemotherapy and radiation, our findings suggest that this signaling pathway represents a key molecular target for anticancer strategies, and that BetA represents a novel transcription factor targeting agent. It is tempting to speculate that combinations of BetA with chemopreventive/therapeutic agents working through different pathways might prove useful in providing new treatment approaches for prostate cancer. Further mechanism-based studies with BetA directing NF-κB signaling pathways in preclinical models of prostate cancer are needed to validate these findings.

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This work was conducted in The James & Eilleen Dicke Research Laboratory, Department of Urology, Case Western Reserve University, Cleveland, Ohio.

Abbreviations

NF-κB	Nuclear Factor-kappaB

EMSA	electrophoretic-mobility shift assay
ALLN	N-acetyl leucyl leucyl norleucinal
Sp	transcription factors specificity protein

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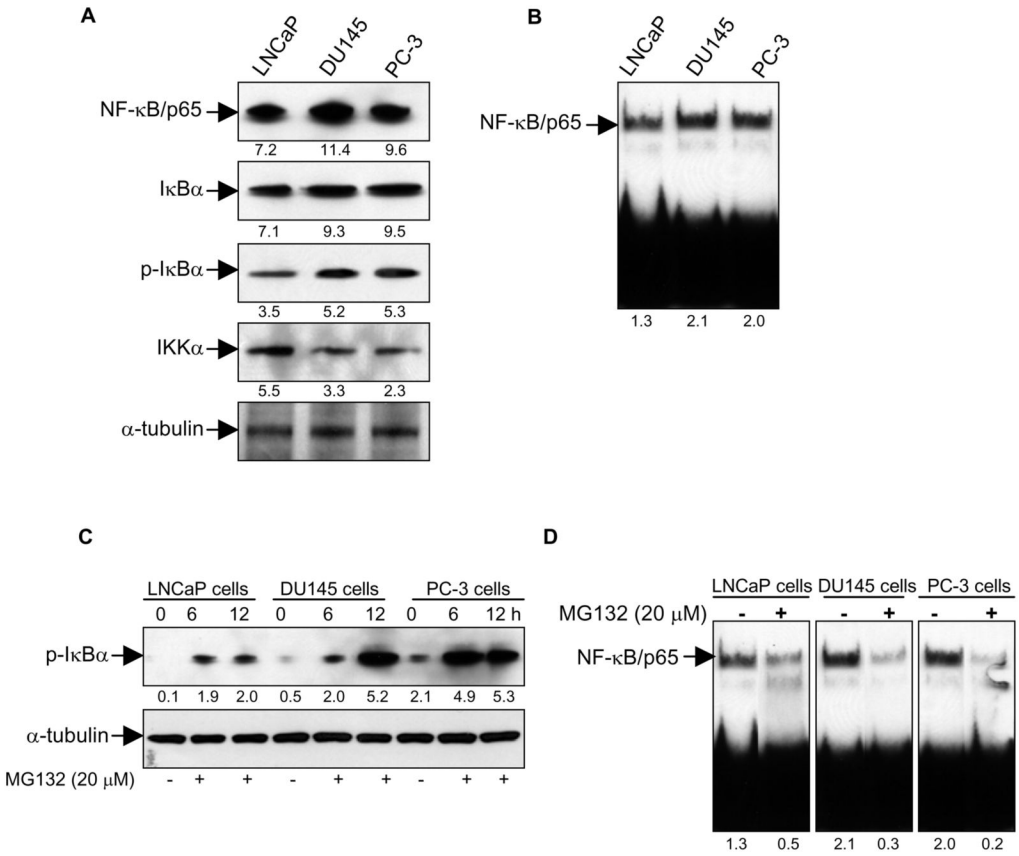

Figure 1. Constitutive NF-κB/p65 activation in human prostate cancer LNCaP, DU145 and PC-3 cells

A, immunoblotting for NF-κB/p65, IKKα, IκBa expression and its phosphorylation in whole cell lysates. ***B,*** DNA binding by EMSA. ***C,*** rate of phosphorylation of IκBα treated with 20 μM MG132 for 6 and 12 h. ***D,*** NF-κB DNA binding activity in all three cell lines treated with 20 μM MG132 for 12 h. The blot was stripped and reprobed with anti-tubulin antibody to ensure equal protein loading. EMSA was performed to identify the nuclear translocation of NF-κB/p65 and its binding to DNA. Data shown below the blots represents densitometry of the bands.

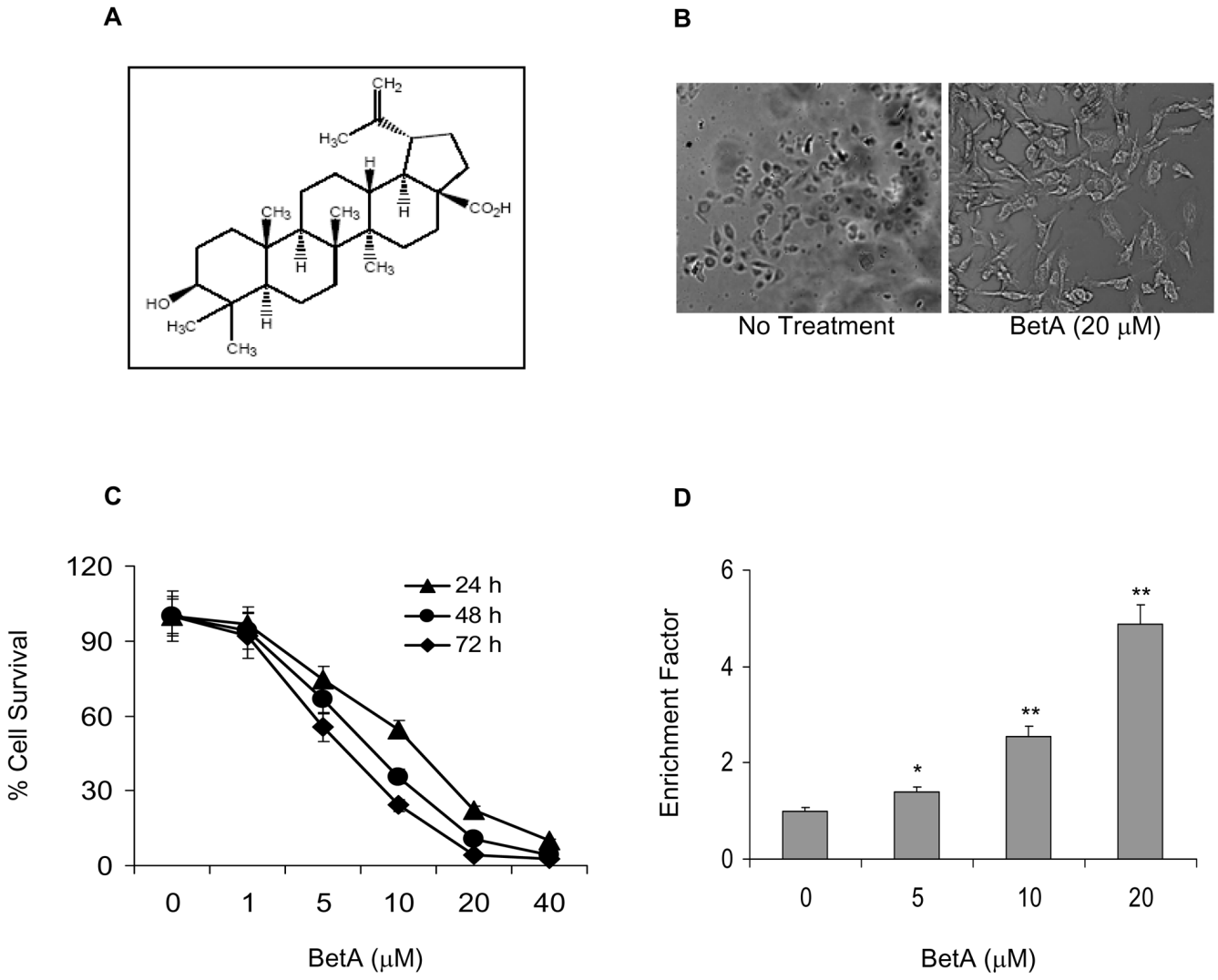

Figure 2. Effect of Betulinic acid on cell survival and apoptosis in human prostate carcinoma PC-3 cells

A, structure of Betulinic acid. ***B,*** microphotograph of cells treated with 20 μM BetA. ***C,*** percentage of cells survived after treatment with BetA for 24, 48 and 72 h with different concentrations as demonstrated by MTT assay. Representative data Mean ± SE, n=8 which was repeated twice with similar results. ***D,*** apoptosis in the lysates from PC-3 cells treated with different concentration of BetA for 24 h, values are represented as enrichment factor described in materials and methods section. Data, Mean ± SE, n=3 significantly different from control, *p<0.05; and **p<0.001.

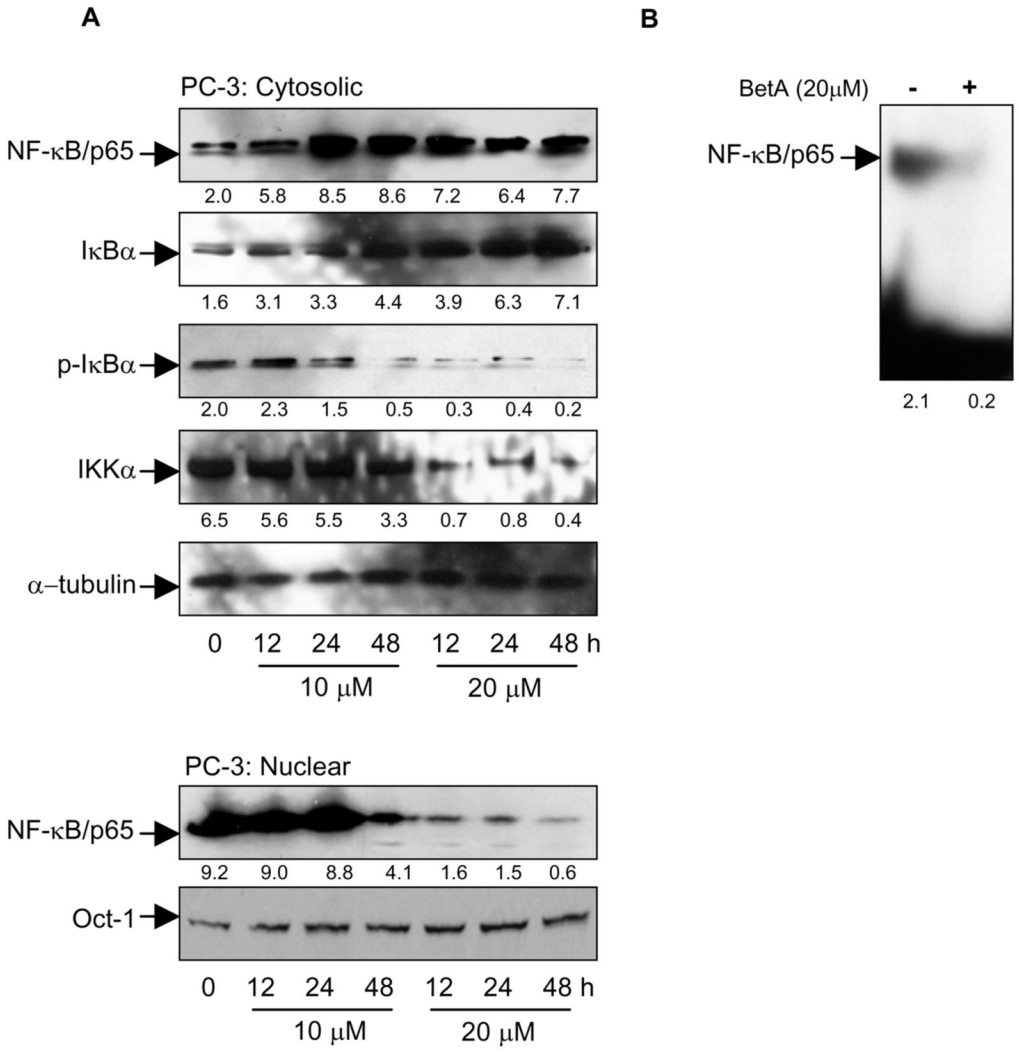

Figure 3. Effect of Betulinic acid on NF-κB signaling pathway in human prostate carcinoma PC-3cells

A, immunoblotting for NF-κB/p65, IκBα, p-IκBα and IKKα in the cytosol and nuclear levels of NF-κB/p65 after treatment of cells with BetA in dose and time dependent manner. The blot was stripped and reprobed with anti-tubulin and anti-Oct-1 antibody to ensure equal protein loading in cytosolic and nuclear fractions. ***B,*** NF-κB/p65 DNA binding activity in PC-3 cells treated with 20 μM BetA for 24 h. EMSA was performed to identify nuclear translocation of NF-κB/p65 its binding to DNA. Data shown below the blots represents densitometry of the bands.

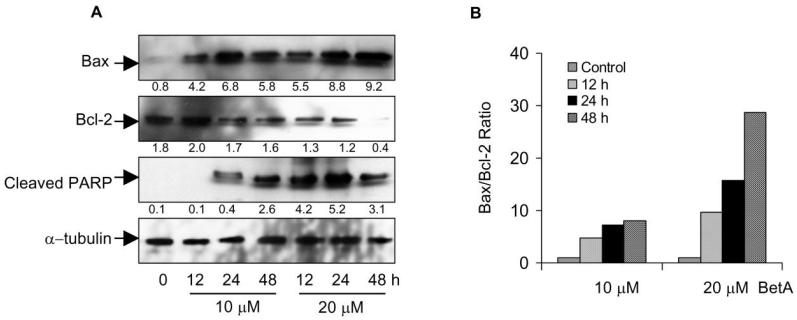

Figure 4. Effect of Betulinic acid on Bcl-2 family member proteins and apoptosis in human prostate cancer PC-3 cells

A, immunoblotting for Bax, Bcl-2, and PARP cleavage using lysates from PC-3 cells treated with various concentration of BetA for indicated time periods. The blot was stripped and reprobed with anti-tubulin antibody to ensure equal protein loading. Data shown below the blots represents densitometry of the bands. ***B,*** Bax/Bcl-2 ratio after densitometric analysis for proteins.

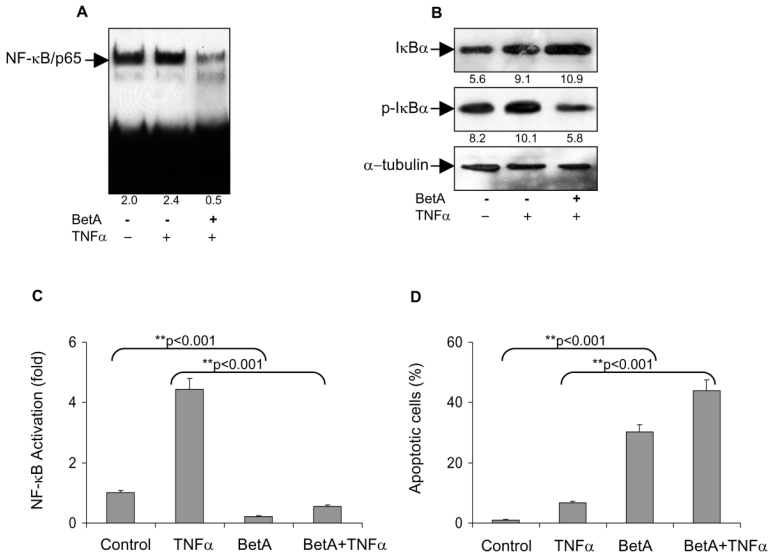

Figure 5. Effect of Betulinic acid on TNFα-induced NF-κB activation and apoptosis in human prostate cancer PC-3 cells

A, NF-κB/p65 DNA binding activity in PC-3 cells pretreated with 20 μM BetA for 16 h followed by TNFα (10 ng/ml) treatment for 30 min. ***B,*** immunoblotting for IκBα and p-IκBα in PC-3 cells after similar treatments. The blot was stripped and reprobed with anti-tubulin antibody to ensure equal protein loading. Data shown below the blots represents densitometry of the bands. ***C,*** NF-κB-dependent reporter gene assay after transient transfection of cells with the NF-κB-regulated reporter construct followed by incubation with 20 μM BetA for 16 h, and then stimulation with 10 ng/ml TNFα for 30 min. ***D,*** estimation of apoptosis by FACS analysis of annexin V-PI stained cells following similar treatment. Data, Mean ± SE, n=3 significantly different from control, **p<0.001.