

Betulinic Acid Suppresses Carcinogen-Induced NF- κ B Activation Through Inhibition of I κ B α Kinase and p65 Phosphorylation: Abrogation of Cyclooxygenase-2 and Matrix Metalloprotease-9

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Betulinic acid (BA), a pentacyclic triterpene isolated from the bark of the white birch tree, has been reported to be a selective inducer of apoptosis in tumor cells. It also exhibits anti-inflammatory and immunomodulatory properties. How BA mediates these effects is not known. Because of the critical role of the transcription factor NF- κ B in growth modulatory, inflammatory, and immune responses, we postulated that BA modulates the activity of this factor. In this study we investigated the effect of BA on NF- κ B and NF- κ B-regulated gene expression activated by a variety of inflammatory and carcinogenic agents. BA suppressed NF- κ B activation induced by TNF, PMA, cigarette smoke, okadaic acid, IL-1, and H₂O₂. The suppression of NF- κ B activation was not cell-type specific. BA suppressed the activation of I κ B α kinase, thus abrogating the phosphorylation and degradation of I κ B α . We found that BA inhibited NF- κ B activated by TNFR 1, TNFR-associated death domain, TNFR-associated factor 2, NF- κ B-inducing kinase, and I κ B α kinase. Treatment of cells with this triterpinoid also suppressed NF- κ B-dependent reporter gene expression and the production of NF- κ B-regulated gene products such as cyclooxygenase-2 and matrix metalloproteinase-9 induced by inflammatory stimuli. Furthermore, BA enhanced TNF-induced apoptosis. Overall, our results indicated that BA inhibits activation of NF- κ B and NF- κ B-regulated gene expression induced by carcinogens and inflammatory stimuli. This may provide a molecular basis for the ability of BA to mediate apoptosis, suppress inflammation, and modulate the immune response. *The Journal of Immunology*, 2003, 171: 3278–3286.

Betulinic acid (BA)² 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 in athymic mice (1, 2). BA is also found in various other plants widespread in tropical regions (e.g., *Tryphillum peltaum*, *Ancistrocladus heyneanus*, *Zizyphus joazeiro*, *Diospyros leucomelas*, *Tetracera boliviana*, and *Syzygium formosanum*). BA has been shown to induce apoptosis in neuroblastomas (3) and glioblastomas (4, 5) through the mitochondrial activation pathway (6, 7). Our study showed that BA has a chemopreventive activity in patients with congenital naevi (8). BA also appears to be active against HIV (9–11), and it has displayed anti-inflammatory activities in various experimental systems (12, 13).

Carcinogenesis typically involves cellular transformation, hyperproliferation, invasion, angiogenesis, and metastasis. These processes are activated by various carcinogens, inflammatory agents, and tumor promoters, such as cigarette smoke, phorbol ester, okadaic acid (OA), H₂O₂, and TNF (14). Although initially discovered through its anticancer activity (15), TNF is now known

to be involved in cellular transformation (16), tumor promotion (17), and induction of metastasis (18–20). In agreement with these observations, mice deficient in TNF have been shown to be resistant to skin carcinogenesis (21). For several tumors, TNF has been shown to be a growth factor (22, 23). Like phorbol ester, TNF mediates these effects in part through activation of a protein kinase C (PKC) pathway (24). Similar to TNF, other inflammatory cytokines have also been implicated in tumorigenesis (25, 26). Thus, agents that can suppress the expression of TNF and other inflammatory agents have chemopreventive potential (27, 28). Most carcinogenic agent and inflammatory agents, including phorbol ester, OA, H₂O₂, and TNF have been shown to activate the transcription factor NF- κ B.

NF- κ B represents a group of five proteins, namely c-Rel, Rel A (p65), Rel B, NF- κ B1 (p50 and p105), and NF- κ B2 (p52) (29). NF- κ B is regulated by an inhibitor called I κ B, a gene family consisting of I κ B α , I κ B β , I κ B ϵ , I κ B γ , Bcl-3, p100, and p105 (29). In an inactive state, NF- κ B is present in the cytoplasm as a heterotrimer consisting of p50, p65, and I κ B α subunits. In response to an activation signal, the I κ B α subunit is phosphorylated at serine residues 32 and 36, ubiquitinated at lysine residues 21 and 22, and degraded through the proteosomal pathway, thus exposing the nuclear localization signals on the p50-p65 heterodimer. The p65 is then phosphorylated, leading to nuclear translocation and binding to a specific sequence in DNA, which in turn results in gene transcription. The phosphorylation of I κ B α is catalyzed by the I κ B α kinase (IKK). The latter consist of IKK- α , IKK- β , and IKK- γ (also called NF- κ B essential modulator) (30). The gene deletion studies have indicated that IKK- β is essential for NF- κ B activation by most agents (30). Which kinase induces the phosphorylation of p65 is controversial, but IKK- β , PKC, and protein kinase A have been implicated (30–32).

NF- κ B has been shown to regulate the expression of a number of genes whose products are involved in carcinogenesis/tumorigenesis (33, 34). These include antiapoptosis genes (e.g., *cIAP*,

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² Abbreviations used in this paper: BA, betulinic acid; PKC, protein kinase C; IKK, I κ B α kinase; NIK, NF- κ B-inducing kinase; TRAF 2, TNFR-associated factor-2; TRADD, TNFR-associated death domain; SEAP, secretory alkaline phosphatase; COX-2, cyclooxygenase-2; MMP-9, matrix metalloprotease-9; CSC, cigarette smoke condensate; OA, okadaic acid.

survivin, *TRAF*, *Bcl-2*, *Bcl-xl*), *cyclooxygenase-2 (COX-2)*, *matrix metalloproteinase-9 (MMP-9)*, adhesion molecules, chemokines, inflammatory cytokines, inducible NO synthase, and cell cycle regulatory genes (e.g., *cyclin D1*). Thus, agents that can suppress NF- κ B activation have the potential to suppress carcinogenesis and thus have therapeutic potential (35–37). Several natural compounds have been reported to have chemopreventive activities (35–38). That plant-derived compounds mediate their chemopreventive effects through the suppression of NF- κ B activation induced by various carcinogens has been proposed (39).

The mechanisms by which BA suppresses cell growth, inhibits cytokine production, and regulates the immune system are not known. Because numerous studies have shown that growth modulatory, antiviral, chemopreventive, and anti-inflammatory activities are mediated through the suppression of a transcription factor NF- κ B activation, and because most carcinogenic agent and inflammatory agents have been shown to activate the transcription factor NF- κ B, we postulated that BA mediates its effects through this transcription factor. In this study we tested this hypothesis. We investigated the effect of BA on NF- κ B activation induced by different carcinogens in different cell lines. We also investigated the step in the NF- κ B activation pathway affected by BA. Additionally we examined the effect of BA on the NF- κ B-dependent reporter gene expression.

Materials and Methods

Reagents

Penicillin, streptomycin, RPMI 1640 medium, and FBS were obtained from Life Technologies (Grand Island, NY). BA was purchased from Alexis (Lausen, Switzerland). PMA, OA, and H₂O₂ were obtained from Sigma-Aldrich (St. Louis, MO). Bacteria-derived human rTNF, purified to homogeneity with a specific activity of 5×10^7 U/mg, was kindly provided by Genentech (South San Francisco, CA). The cigarette smoke condensate (CSC) was kindly provided by Dr. C. G. Gariola (University of Kentucky, Lexington, KY). The polyclonal Abs anti-p65, anti-p50, anti-I κ B α , and anti-cyclin D1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phospho-specific anti-I κ B α (Ser³²) Ab was purchased from Cell Signaling (Beverly, MA). Anti-IKK- α and anti-IKK- β Abs were kindly provided by Imgenex (San Diego, CA). The polyclonal Ab, which recognizes the serine 529 phosphorylated form of p65, was obtained from Rockland (Gilbertsville, PA).

Cell lines

Epithelial cell line HCT 116 and Caco 2 cells (colon carcinoma), and H 1299 cells (lung adenocarcinoma) were obtained from American Type Culture Collection (Manassas VA). All cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin.

EMSA

To determine NF- κ B activation, we performed EMSA as described (40, 41). Briefly, nuclear extracts prepared from TNF-treated cells (1×10^6 /ml) were incubated with ³²P-end-labeled, 45-mer, double-stranded NF- κ B oligonucleotide (15 μ g of protein with 16 fmol of DNA) from the HIV long-terminal repeat, 5'-TTGTTACAAGGGACTTCCGCTGGGGACTTTC CAGGGAGGCGTGG-3' (bold indicates NF- κ B binding sites) for 30 min at 37°C. The DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5'-TTGTTACAACACTACTTTC CGTGCTCACT **TTCCAGGGAGGCGTGG**-3', was used to examine the specificity of binding of NF- κ B to the DNA. The specificity of binding was also examined by competition with the unlabeled oligonucleotide. For supershift assays, nuclear extracts prepared from TNF-treated cells were incubated with Abs against either the p50 or p65 subunits of NF- κ B for 30 min at 37°C, and then the complex was analyzed by EMSA. Abs against cyclin D1 and preimmune serum were included as negative controls. The dried gels were visualized, and radioactive bands were quantitated by a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using Imagequant software.

Western blot analysis

To determine the levels of protein expression in the cytoplasm or the nucleus, we prepared extracts (43) from TNF-treated cells and fractionated

them by SDS-PAGE. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, blotted with each Ab, and detected by ECL reagent (Amersham Pharmacia Biotech, Piscataway, NJ). The bands obtained were quantitated using Personal Densitometer Scan version 1.30 using Imagequant software version 3.3 (Molecular Dynamics).

IKK assay

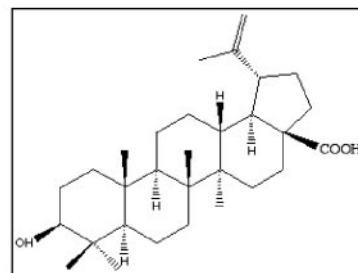
The IKK assay was performed by a method described previously (43). Briefly, IKK complex from whole-cell extract was precipitated with Ab against IKK- α , followed by treatment with protein A/G-Sepharose beads (Pierce, Rockford, IL). After a 2-h incubation, the beads were washed with lysis buffer and then assayed in kinase assay mixture containing 50 mM HEPES (pH 7.4), 20 mM MgCl₂, 2 mM DTT, 20 μ Ci [γ -³²P]ATP, 10 μ M unlabeled ATP, and 2 μ g of substrate GST-I κ B α (1–54). After incubation at 30°C for 30 min, the reaction was terminated by boiling with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-PAGE, the gel was dried, and the radioactive bands were visualized by PhosphorImager. To determine the total amounts of IKK- α and IKK- β in each sample, whole-cell extracts were immunoprecipitated with Ab against IKK- α , resolved on 7.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with either anti-IKK- α or anti-IKK- β Abs.

NF- κ B-dependent reporter secretory alkaline phosphatase (SEAP) expression assay

The effect of BA on TNF-, TNFR-, TNFR-associated death domain (TRADD)-, TNFR-associated factor-2 (TRAF 2)-, NF- κ B-inducing kinase (NIK)-, IKK, and p65-induced NF- κ B-dependent reporter gene transcription was analyzed by SEAP assay as previously described (44). Briefly, HCT 116 cells (5×10^5 cells/well) were plated in six-well plates and transiently transfected by the calcium phosphate method with pNF- κ B-SEAP (0.5 μ g). To examine TNF-induced reporter gene expression, we transfected the cells with 0.5 μ g of the SEAP expression plasmid and 2 μ g of the control plasmid pCMVFLAG1 DNA for 24 h. Thereafter, the cells were treated for 24 h with 30 μ M BA and then stimulated with TNF for 24 h. The cell culture medium was then harvested and analyzed for alkaline phosphatase (SEAP) activity according to protocol, essentially as described by the manufacturer (Clontech Laboratories, Palo Alto, CA), using a 96-well fluorescence plate reader (Fluoroscan II; Labsystems, Chicago, IL) with excitation set at 360 nm and emission at 460 nm.

Immunocytochemistry for NF- κ B p65 localization

The effect of BA on the nuclear translocation of p65 was examined by an immunocytochemical method as described previously (45). Briefly, treated cells were plated on a poly L-lysine-coated glass slide by centrifugation using a cytospin 4 (Thermoshendon, Pittsburg, PA), air-dried, fixed with cold acetone, and permeabilized with 0.2% Triton X-100. After being washed in PBS, slides were blocked with 5% normal goat serum for 1 h and then incubated with rabbit polyclonal anti-human p65 Ab at a 1/100 dilution. After overnight incubation at 4°C, the slides were washed, incubated with goat anti-rabbit IgG-Alexa 594 at a 1/100 dilution for 1 h, and counterstained for nuclei with Hoechst 33342 (50 ng/ml) for 5 min. Stained slides were mounted with mounting medium, purchased from Sigma-Aldrich, and analyzed under a fluorescence microscope (Labophot-2; Nikon, Tokyo, Japan). Pictures were captured using Photometrics Coolsnap CF color camera (Nikon) and MetaMorph version 4.6.5 software (Universal Imaging, Downingtown, PA).



Betulinic acid [3 β -Hydroxy-20(29)-lupaene-28-oic acid]

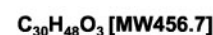


FIGURE 1. Chemical structure of BA.

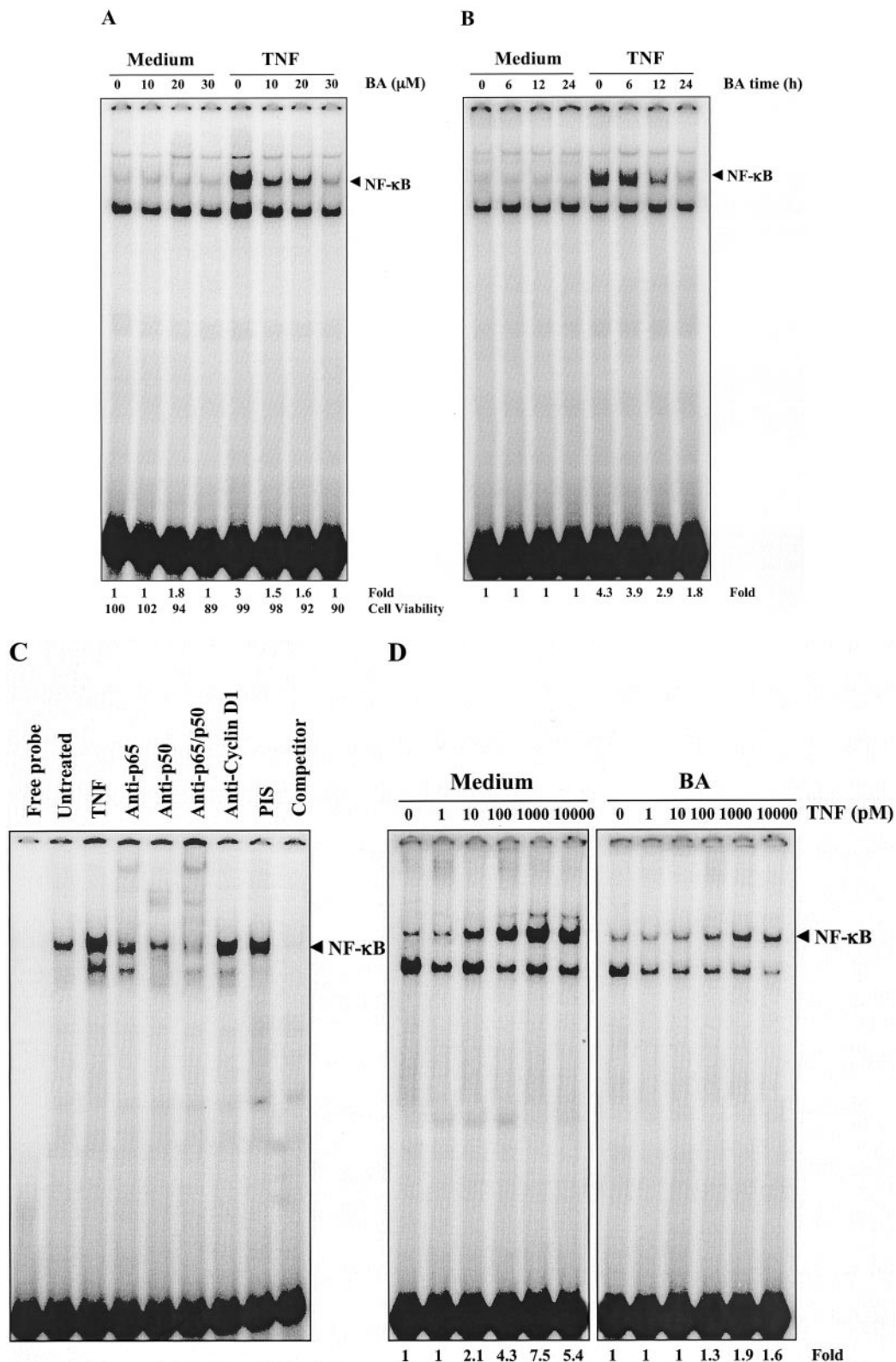


FIGURE 2. Effect of BA on TNF-induced NF- κ B activation in HCT 116 cells. **A**, Dose response of BA for the inhibition of TNF-induced NF- κ B activation. Cells were preincubated with different concentrations of BA, followed by an incubation with 0.1 nM TNF. After these treatments, nuclear extracts were prepared and then assayed for NF- κ B activation by EMSA, as described in *Materials and Methods*. Cell viability was determined by MTT assay. **B**, Time-dependent inhibition of TNF-induced NF- κ B activation by BA. Cells were preincubated with 30 μ M BA for the indicated times, treated with 0.1 nM TNF, and then subjected to EMSA. **C**, NF- κ B induced by TNF is composed of p65 and p50 subunits. Nuclear extracts from untreated or TNF-treated cells were incubated with different Abs or unlabeled NF- κ B oligoprobe and then assayed for NF- κ B activation by EMSA. **D**, Effect of BA on the activation of NF- κ B induced by different concentrations of TNF. Cells were incubated with 30 μ M BA, treated with different concentrations of TNF, and then subjected to EMSA for NF- κ B activation. The results shown are representative of two or three independent experiments.

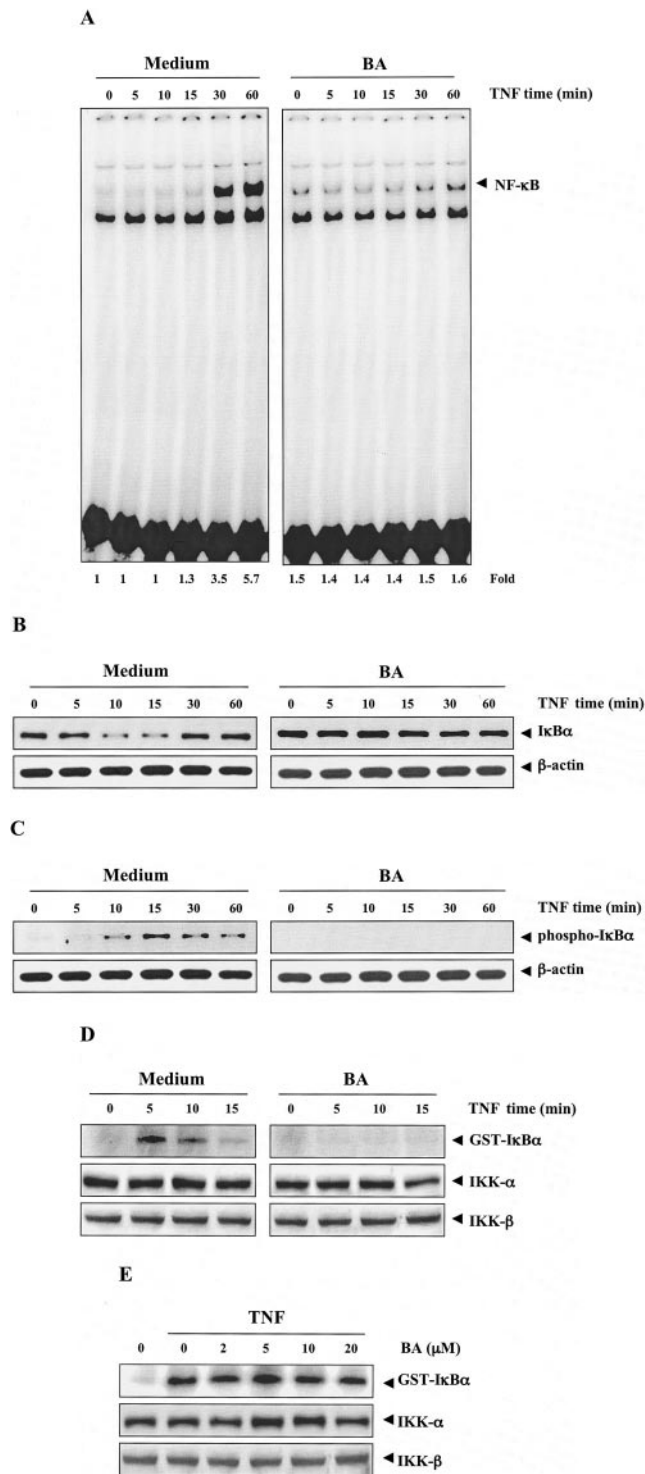


FIGURE 3. Effect of BA on the I κ B α phosphorylation and degradation induced by TNF in HCT 116 cells. *A*, TNF induces time-dependent activation of NF- κ B. Cells were incubated with 30 μ M BA, treated with 0.1 nM TNF for the times indicated, and then analyzed for NF- κ B activation by EMSA. *B*, Effect of BA on TNF-induced degradation of I κ B α . Cells were incubated with 30 μ M BA and treated with 0.1 nM TNF for the indicated times. Cytoplasmic extracts were prepared, fractionated on 10% SDS-PAGE, and electrotransferred. Western blot analysis was used with anti-I κ B α Ab. Anti- β -actin Ab was the loading control. *C*, Effect of BA on the phosphorylation of I κ B α by TNF. Cells were incubated with 30 μ M BA, and treated with 0.1 nM TNF for the indicated times. Cytoplasmic extracts were fractionated and then blotted using phosphospecific anti-I κ B α Ab. *D*, Effect of BA on the activation of IKK by TNF. Cells were incubated with 30 μ M BA and then activated with 1 nM TNF for different

TUNEL assay

TNF-induced apoptosis was determined by TUNEL assay using in situ Cell Death Detection reagent (Roche, Basel Switzerland). Briefly, 1×10^5 cells were incubated with 30 μ M BA for 24 h, and then treated with 1 nM TNF for 16 h at 37°C. Thereafter, cells were air-dried, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100 in 0.1% sodium citrate. After washing, cells were incubated with reaction mixture for 60 min in 37°C. Stained cells were mounted with mounting medium (Sigma-Aldrich) and analyzed under a fluorescence microscope (Labophot-2). TUNEL positive cells were defined as apoptotic cells.

Results

In this study, we examined the effect of BA on NF- κ B activation induced by various carcinogens and inflammatory stimuli, including phorbol ester, OA, H₂O₂, IL-1, and TNF. The effect of BA on TNF-induced NF- κ B activation was studied in detail, because this pathway has been well characterized. The concentration of BA, the NF- κ B activators used, and the time of exposure had minimal effect on the viability of these cells (data not shown). The structure of BA used in our study [3 β -hydroxy-20(29)-lupaene-28-oic acid] is shown in Fig. 1.

BA inhibits TNF-dependent NF- κ B activation

We first examined the effect of BA on TNF-induced NF- κ B activation. We used colon cancer cell lines (HCT 116 and Caco 2) for most studies, because NF- κ B has been shown to play a major role in colon carcinogenesis. HCT 116 cells were preincubated for 24 h with different concentrations of BA, and treated with 0.1 nM TNF for 30 min at 37°C; nuclear extracts were then prepared and analyzed for NF- κ B activation by EMSA. As shown in Fig. 2*A*, BA inhibited TNF-mediated NF- κ B activation in a dose-dependent manner, with maximum inhibition ($91.5 \pm 4.9\%$) occurring at 30 μ M. BA by itself did not induce NF- κ B activation.

We next incubated cells with BA for different times, and then exposed them to TNF for 30 min. TNF-induced NF- κ B activation was inhibited by BA in a time-dependent manner. When we incubated cells with 30 μ M BA for 24 h, NF- κ B activation by TNF was inhibited by $81.4 \pm 7.9\%$ (Fig. 2*B*).

Because NF- κ B is a complex of proteins, various combinations of Rel/NF- κ B protein can constitute an active NF- κ B heterodimer that binds to a specific sequence in the DNA (24). To show that the retarded band visualized by EMSA in TNF-treated cells was indeed NF- κ B, we incubated nuclear extracts from TNF-stimulated cells with Abs to either the p50 (NF- κ B1) or the p65 (Rel A) subunit of NF- κ B. Both shifted the band to a higher molecular mass (Fig. 2*C*), thus suggesting that the TNF-activated complex consisted of p50 and p65 subunits. Neither preimmune serum nor the irrelevant Ab anti-cyclin D1 had any effect. Excess unlabeled NF- κ B (100-fold) caused complete disappearance of the band.

Previous studies from our laboratory have shown that 10 nM TNF can activate NF- κ B within 5 min, and that this induction is higher in its intensity than that obtained with cells using 100-fold lower concentrations of TNF for longer times (46). To determine the effect of BA on NF- κ B activation at higher concentrations of

times. Whole-cell extracts were immunoprecipitated with Ab against IKK- α and analyzed by immunocomplex kinase assay. To examine the effect of BA on the level of expression of IKK proteins, whole-cell extracts were immunoprecipitated with Ab against IKK- α , and Western blot analysis was performed using anti-IKK- α and anti-IKK- β Abs. *E*, Direct effect of BA on the activation of IKK induced by TNF. Whole-cell extracts were prepared from 1 nM TNF-treated cells, and immunoprecipitated with Ab against IKK- α . Thereafter, immunocomplex kinase assay was performed in the absence or presence of the indicated concentration of BA. The results shown are representative of three independent experiments.

TNF, cells were treated with concentrations of TNF varying from 1 to 10,000 pM for 30 min in the absence or presence of BA, and then NF- κ B activation was analyzed by EMSA (Fig. 2D). TNF at a concentration of 10 nM activated NF- κ B activity strongly, but in cells treated with BA, 10 nM TNF did not activate NF- κ B ($84.2 \pm 3.1\%$ inhibition). These results show that BA is a very potent inhibitor of TNF-induced NF- κ B activation.

BA inhibits TNF-dependent I κ B α phosphorylation

The translocation of NF- κ B to the nucleus is preceded by the phosphorylation, ubiquitination, and proteolytic degradation of I κ B α (29). To determine whether inhibition of TNF-induced NF- κ B activation was caused by inhibition of I κ B α degradation, we pre-treated cells with BA for 24 h and then exposed them to 0.1 nM TNF for different times. We then examined the cells for NF- κ B in the nucleus by EMSA and for I κ B α status in the cytoplasm by Western blot analysis. NF- κ B was increasingly activated with increases in TNF incubation times. BA pretreatment dramatically decreased NF- κ B activation ($89.6 \pm 3.4\%$) even after 60 min of TNF stimulation (Fig. 3A). Whereas TNF induced I κ B α degradation quickly in control cells, no I κ B α degradation was evident in BA-pretreated cells (Fig. 3B). Thus, BA inhibited both TNF-induced NF- κ B activation and I κ B α degradation.

To determine whether BA blocks TNF-induced I κ B α phosphorylation, we assayed the TNF-induced phosphorylated form of I κ B α by Western blot analysis using Ab specific for the serine-phosphorylated form of I κ B α . Although TNF induced I κ B α phos-

phorylation quickly, BA almost completely suppressed this phosphorylation.

BA inhibits TNF-induced IKK activation

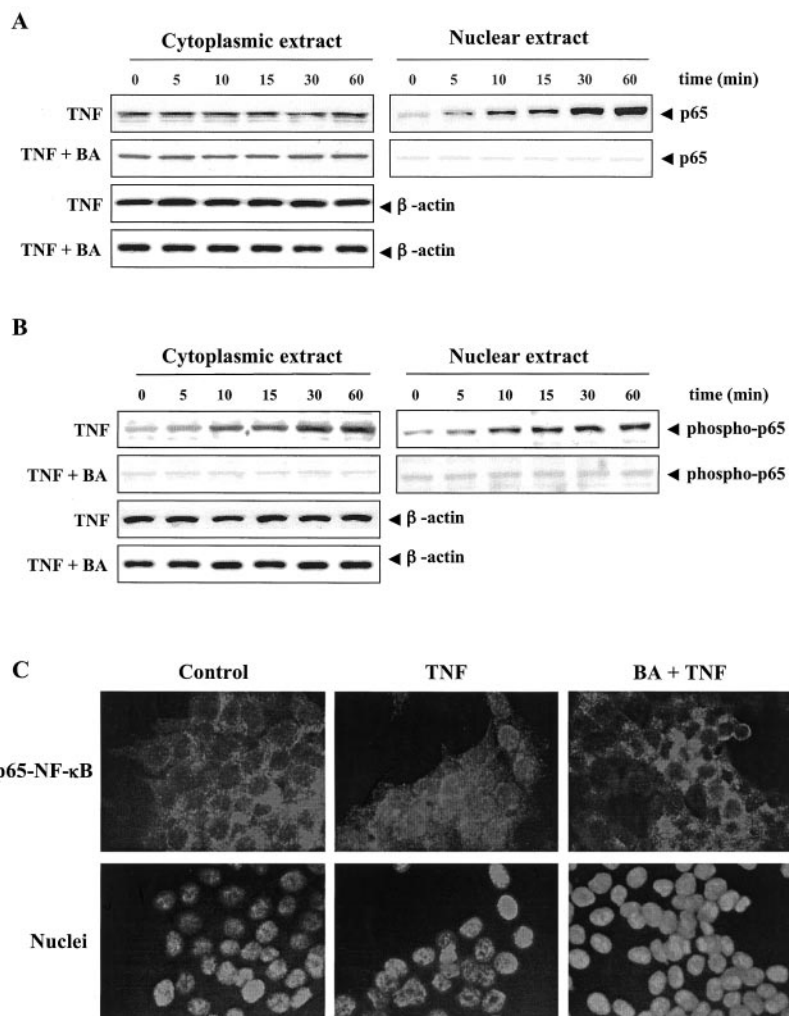
We next determined the effect of BA on TNF-induced IKK activation, because IKK is required for TNF-induced I κ B α phosphorylation (29). Immune complex kinase assays showed that TNF activated IKK as early as 5 min after TNF treatment, whereas BA treatment completely suppressed this activation (Fig. 3D). TNF or BA had no effect on the expression of either IKK- α or IKK- β proteins.

To evaluate whether BA binds directly to IKK proteins, we incubated whole-cell extracts from untreated cells and those treated with TNF for 5 min with various concentrations of BA. The kinase assay results (Fig. 3E) showed that BA did not directly affect the activity of IKK, suggesting that BA inhibits TNF-induced IKK activity by an indirect mechanism.

BA inhibits TNF-induced nuclear translocation of p65

We also analyzed the effect of BA on TNF-induced phosphorylation and nuclear translocation of p65. Western blot analysis showed that TNF induced nuclear translocation of p65 in a time-dependent manner. As early as 5 min after TNF stimulation, p65 was translocated to the nucleus, and nuclear concentrations increased for up to 60 min. However, BA preincubation prevented TNF-induced nuclear translocation of p65 (Fig. 4A). TNF induced

FIGURE 4. Effect of BA on the translocation of p65 into nuclear induced by TNF in HCT 116 cells. *A*, Western blot analysis of p65 using cytoplasmic and nuclear extract. Cells were incubated with 30 μ M BA and treated with 0.1 nM TNF for the indicated times. Cytoplasmic and nuclear extracts were prepared and analyzed by Western blot analysis using anti-p65 Ab. *B*, Effect of BA on the phosphorylation of p65 by TNF in cytosol and nuclear. Cells were incubated with 30 μ M BA and treated with 0.1 nM TNF for the indicated times. Cytoplasmic and nuclear extracts were prepared and analyzed by Western blot analysis using phospho-specific anti-p65 Ab. *C*, Immunocytochemical analysis of p65 localization after treatment with 1 nM TNF in the absence or presence of 30 μ M BA. Cells were incubated with BA and then treated with 1 nM TNF. Cells were subjected to immunocytochemistry as described in *Materials and Methods*. The results shown are representative of two or three independent experiments.



the phosphorylation of p65 in a time-dependent manner, whereas BA suppressed it almost completely (Fig. 4B).

Immunocytochemistry confirmed that in untreated cells, p65 was localized in the cytoplasm; in TNF-treated cells, it was translocated into the nucleus; and in BA-treated cells, TNF-induced p65 translocation to the nucleus was clearly suppressed (Fig. 4C).

BA blocks NF-κB activation induced by PMA, H₂O₂, IL-1β, OA, and CSC

PMA, H₂O₂, IL-1β, OA, and CSC are potent activators of NF-κB, but the mechanisms by which these agents activate NF-κB differ (33, 34). We found that BA suppressed the NF-κB activation by 90.3 ± 5.9%, 93.1 ± 2%, 78.7 ± 3.7%, 80.4 ± 7.6%, and 74.8 ± 4.2% induced by PMA, H₂O₂, IL-1β, OA, and CSC, respectively (Fig. 5A). These results suggest that the BA acts at a step in the NF-κB activation pathway that is common to all these agents.

Inhibition of NF-κB activation by BA is not cell type specific

That distinct signal transduction pathways could mediate NF-κB induction in different cells has been demonstrated (47). Therefore, we also investigated whether BA could block TNF-induced NF-κB activation in another colon cancer cell line (Caco 2) and in human lung adenocarcinoma cell line (H 1299). The cells were pretreated

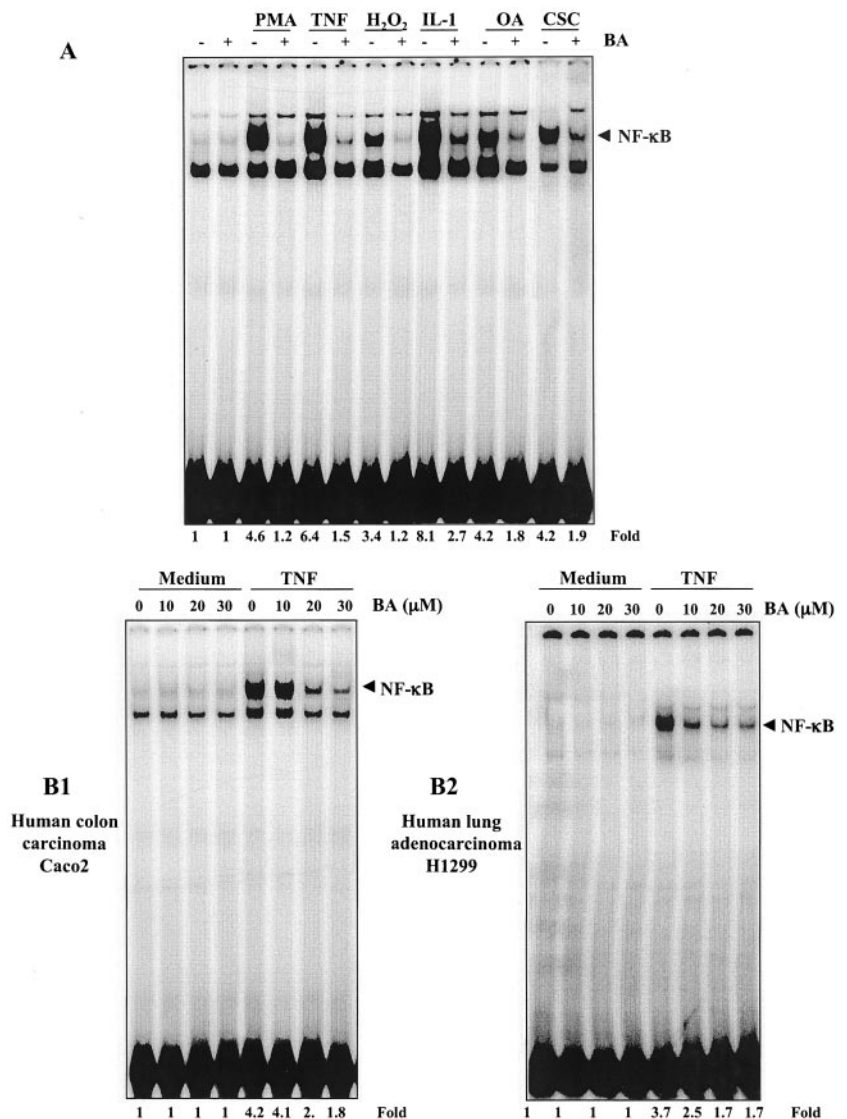
with different concentrations of BA, and then treated with TNF for 30 min. BA completely inhibited most of the TNF-induced NF-κB activation in both cell types (Fig. 5B), indicating a lack of cell-type specificity.

BA represses TNF-induced NF-κB-dependent reporter gene expression

Although we have shown by EMSA that BA blocks NF-κB activation, DNA binding does not always correlate with NF-κB-dependent gene transcription, suggesting there are additional regulatory steps (48). To determine the effect of BA on TNF-induced NF-κB-dependent reporter gene expression, we transiently transfected the cells with the NF-κB-regulated SEAP reporter construct, incubated them with BA, and then stimulated the cells with TNF. An almost 5-fold increase in SEAP activity over the vector control was observed upon stimulation with 1 nM TNF, and BA completely suppressed the TNF-induced stimulation (Fig. 6A). These results demonstrate that BA also represses NF-κB-dependent reporter gene expression induced by TNF.

TNF-induced NF-κB activation is mediated through sequential interaction of the TNFR with TRADD, TRAF 2, NIK, and IKK, resulting in phosphorylation of IκBα (49, 50). To delineate the site of action of BA in the TNF-signaling pathway leading to NF-κB

FIGURE 5. Effect of BA on NF-κB activated by different activators and in different cell lines. *A*, BA blocks NF-κB activation induced by IL-1, PMA, H₂O₂, OA, and CSC. Cells were preincubated with 30 μM BA, treated with 0.1 nM TNF, 10 ng/ml PMA, 250 μM H₂O₂, 500 nM OA, or 1 μg/ml CSC and then analyzed for NF-κB activation as described in *Materials and Methods*. *B*, BA suppresses TNF-induced NF-κB in different cell lines. Caco 2 and H 1299 cells were incubated with 30 μM BA and then treated with 0.1 nM TNF. Nuclear extracts were prepared and analyzed for NF-κB activation as described in *Materials and Methods*. The results shown are representative of three independent experiments.



A

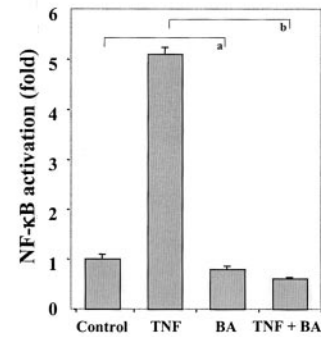
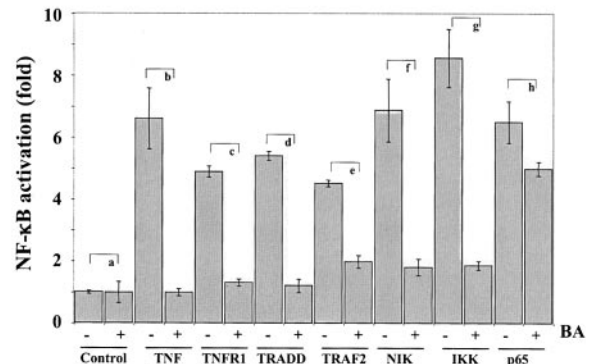
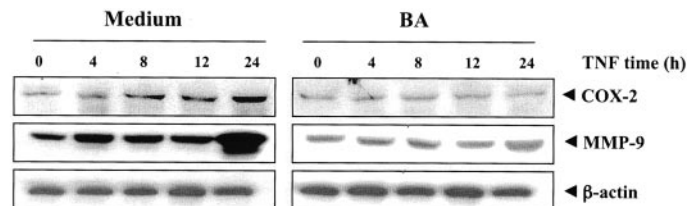


FIGURE 6. BA inhibits the TNF-induced expression of NF- κ B-dependent gene. **A**, HCT 116 cells were transiently transfected with NF- κ B-containing plasmid linked to the *SEAP* gene, and incubated with 30 μ M BA. Cells were treated with 1 nM TNF, and supernatants were collected and assayed for SEAP activity as described in *Materials and Methods*. ^a $p > 0.1$ compared with control cells; ^b $p < 0.001$ compared with TNF-treated cells. The results were the mean of experiments performed in triplicate. **B**, BA inhibits the NF- κ B-dependent reporter gene expression induced by TNF, TNFR 1, TRADD, TRAF 2, NIK, and IKK. Cells were transiently transfected with a NF- κ B-containing plasmid along with indicated plasmids. The supernatants of the culture medium were assayed for SEAP activity as described in *Materials and Methods*. Results are expressed as fold activity of the vector control. ^a $p > 0.1$, ^b $p < 0.005$, ^c $p < 0.001$, ^d $p < 0.001$, ^e $p < 0.01$, ^f $p < 0.005$, ^g $p < 0.005$, ^h $p < 0.05$. The results were the mean of triplicate experiments. **C**, BA inhibits TNF-induced COX-2 and MMP-9 expression. Cells were incubated with 30 μ M BA and then treated with 1 nM TNF for the indicated times. Whole-cell extracts were prepared and analyzed by Western blot analysis using Abs against COX-2 and MMP-9. The results shown are representative of three independent experiments.

B



C



activation, we transfected cells with *TNFR 1*-, *TRADD*-, *TRAF 2*-, *NIK*-, *IKK*-, and *p65*-expressing plasmids, and then monitored NF- κ B-dependent SEAP expression. As shown in Fig. 6B, all of the plasmid-transfected cells induced NF- κ B-*SEAP* gene expression. BA suppressed *TNFR 1*-, *TRADD*-, *TRAF 2*-, *NIK*-, and *IKK*-induced, but not *p65*-induced, NF- κ B reporter gene expression. These results suggested that BA affects NF- κ B activation at a step upstream from p65.

Besides NF- κ B, *COX-2* and *MMP-9*, which have NF- κ B binding sites in their promoters, are also induced by TNF (33–35, 51). Whether BA inhibits TNF-induced *COX-2* and *MMP-9* was examined. Cells were pretreated with BA for 24 h, and then treated with TNF for different times. Whole-cell extracts were prepared and analyzed in 30 μ g of protein by Western blot analysis for *COX-2* and *MMP-9* expression (Fig. 6C). TNF induced both *COX-2* and *MMP-9* expression in a time-dependent manner, and BA blocked TNF-induced expression of *COX-2* and *MMP-9*. The results further strengthen the validity of our postulate, that BA has a role in blocking TNF-induced NF- κ B activation.

BA enhances TNF-induced apoptosis

Activation of NF- κ B has been shown to inhibit, and suppression of NF- κ B has been shown to stimulate TNF-induced apoptosis (22, 52–54). Whether suppression of NF- κ B by BA affects TNF-induced apoptosis was investigated by TUNEL assay. As shown in Fig. 7, pretreatment of cells with BA enhanced the TNF-induced

apoptosis. These results demonstrate a reciprocal relationship between TNF-induced NF- κ B activation and apoptosis.

Discussion

The growth modulatory, antiviral, chemopreventive, and anti-inflammatory activities of BA prompted us to propose the hypothesis that it mediates its effects through the suppression of the transcription factor NF- κ B activation. In this report, we demonstrate that treatment of cells with BA blocks TNF-mediated NF- κ B activation. BA inhibited activation, not only by TNF but also by other inflammatory and carcinogenic agents. The inhibitory effect of BA was not cell-line specific. The inhibitory activity correlated with the suppression of TNF-induced IKK activation, *I κ B α* phosphorylation and degradation, p65 phosphorylation and nuclear translocation, and NF- κ B-dependent reporter gene transcription. BA also inhibited the TNF-induced expression of *COX-2* and *MMP-9*, which have NF- κ B binding sites in their promoters, and regulated their transcription. Furthermore, BA enhanced TNF-induced apoptosis.

There are various ways that BA might inhibit TNF-induced NF- κ B activation. Because NF- κ B activation induced by highly diverse stimuli, including cigarette smoke, TNF, H_2O_2 , LPS, PMA, IL-1, and OA, was inhibited, BA must suppress activation at a step common to all these activators. In response to most of these stimuli, NF- κ B activation requires sequential phosphorylation at

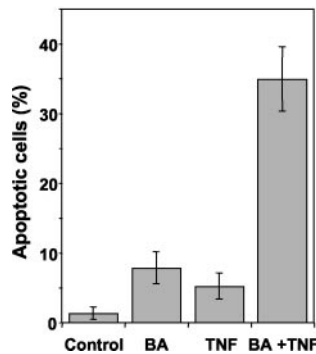


FIGURE 7. BA enhances TNF-induced cell death. Cells were pretreated with 30 μ M BA for 24 h, and then incubated with 1 nM TNF for 16 h. Thereafter, cells were analyzed by TUNEL assay. Data presented are the mean of triplicates.

serines 32 and 36 of $\text{I}\kappa\text{B}\alpha$. In addition, $\text{I}\kappa\text{B}\alpha$ undergoes phosphorylation by activation of a kinase complex consisting of $\text{IKK}\alpha$, $\text{IKK}\beta$, and $\text{IKK}\gamma$ (55, 56), which leads to ubiquitination at lysines 21 and 22, and its eventual degradation (57, 58). Thus, BA must act at a step upstream of $\text{I}\kappa\text{B}\alpha$ phosphorylation.

What steps in the NF- κ B activation pathway that BA inhibits were also examined? The sequential interaction of the NF receptor with TRADD, TRAF 2, and NIK, which then activates IKK (49, 50), is involved. We found that BA inhibits NF- κ B-dependent reporter gene expression induced by TNFR 1, TRADD, TRAF 2, NIK, and IKK, but not by p65, which suggests that BA acts at a step downstream of NIK and upstream from p65. In the present studies, we found that BA inhibited TNF-induced activation of IKK. By using Abs that specifically detect the phosphorylated form of $\text{I}\kappa\text{B}\alpha$, we showed that BA blocks TNF-induced phosphorylation of $\text{I}\kappa\text{B}\alpha$. The phosphorylation of $\text{I}\kappa\text{B}\alpha$ is regulated by IKK (which consists of $\text{IKK}\alpha$, $\text{IKK}\beta$, and $\text{IKK}\gamma$), which in turn is regulated by many upstream kinases, including NIK, TGF β -activated kinase 1, Akt, and mitogen-activated protein kinase kinase 1 (30–34). Akt and NIK are known primarily to activate $\text{IKK}\alpha$, whereas mitogen-activated protein kinase kinase 1 and atypical PKC activate $\text{IKK}\beta$. BA inhibited IKK activity without directly interfering with the IKK protein, thereby blocking $\text{I}\kappa\text{B}\alpha$ phosphorylation and degradation. Thus, it may be possible that BA blocked IKK activation by inhibiting one or many of the upstream kinases responsible for IKK activation.

We found that suppression of NF- κ B activation by BA was observed in a variety of different cell lines, including colon cancer (HCT 116 and Caco 2) and a lung cancer (H 1299). These results indicated that BA is a broad inhibitor of NF- κ B activation. BA has been reported to be a melanoma-specific cytotoxic agent (1, 2). It exhibits cytotoxic activity also against neuroectodermal (3, 5, 6) and brain tumor cells (4). Because NF- κ B is constitutively active in numerous tumors, including melanoma (59), and in brain tumors (60), and because NF- κ B activation has an antiapoptotic role, it is possible that the apoptotic effects of BA are mediated through the down-regulation of NF- κ B, as described in this study. Our results do indeed show that BA can potentiate the TNF-induced apoptosis mediated through the down-regulation of NF- κ B.

BA has also been shown to have chemopreventive effects (8). Because suppression of NF- κ B has been implicated in chemoprevention (35, 39), it is possible that chemopreventive effects of BA are also mediated through NF- κ B. This is in agreement with the mechanism for inhibition of carcinogenesis by naturally occurring and synthetic compounds reported previously (35–40). Several genes known to be involved in tumor promotion are regulated by

NF- κ B, including various growth factors, COX-2, MMP-9, and cell surface adhesion molecules (33, 34).

It is likewise possible that the anticarcinogenic effects of BA are mediated through the suppression of NF- κ B-dependent gene expression. The anti-inflammatory effects of BA could be mediated through suppression of TNF, which has been implicated in inflammation, tumor promotion, and metastasis. As replication of certain viruses such as HIV is also dependent on NF- κ B (33, 34), suppression of HIV replication by BA (9–11) may also be mediated through suppression of NF- κ B. Thus, overall our results suggest that BA may have applications for various diseases mediated through NF- κ B activation, including cancer, inflammation, and AIDS. These possibilities require further investigation.

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