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Ursolic Acid Inhibits Nuclear Factor- κ B Activation Induced by Carcinogenic Agents through Suppression of I κ B α Kinase and p65 Phosphorylation: Correlation with Down-Regulation of Cyclooxygenase 2, Matrix Metalloproteinase 9, and Cyclin D1¹

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ABSTRACT

The process of tumorigenesis requires cellular transformation, hyperproliferation, invasion, angiogenesis, and metastasis. Several genes that mediate these processes are regulated by the transcription factor nuclear factor- κ B (NF- κ B). The latter is activated by various carcinogens, inflammatory agents, and tumor promoters. Thus, agents that can suppress NF- κ B activation have the potential to suppress carcinogenesis. Ursolic acid, a pentacyclic triterpene acid, has been shown to suppress the expression of several genes associated with tumorigenesis, but whether ursolic acid mediates its effects through suppression of NF- κ B is not understood. In the study described in the present report, we found that ursolic acid suppressed NF- κ B activation induced by various carcinogens including tumor necrosis factor (TNF), phorbol ester, okadaic acid, H₂O₂, and cigarette smoke. These effects were not cell type specific. Ursolic acid inhibited DNA binding of NF- κ B consisting of p50 and p65. Ursolic acid inhibited I κ B α degradation, I κ B α phosphorylation, I κ B α kinase activation, p65 phosphorylation, p65 nuclear translocation, and NF- κ B-dependent reporter gene expression. Ursolic acid also inhibited NF- κ B-dependent reporter gene expression activated by TNF receptor, TNF receptor-associated death domain, TNF receptor-associated factor, NF- κ B-inducing kinase, I κ B α kinase, and p65. The inhibition of NF- κ B activation correlated with suppression of NF- κ B-dependent cyclin D1, cyclooxygenase 2, and matrix metalloproteinase 9 expression. Thus, overall, our results indicate that ursolic acid inhibits I κ B α kinase and p65 phosphorylation, leading to the suppression of NF- κ B activation induced by various carcinogens. These actions of ursolic acid may mediate its antitumorigenic and chemosensitizing effects.

INTRODUCTION

Tumorigenesis is a process that requires cellular transformation, hyperproliferation, invasion, angiogenesis, and metastasis. This process is activated by various carcinogens (such as cigarette smoke), inflammatory agents (such as TNF³ and H₂O₂), and tumor promoters (such as phorbol ester and okadaic acid; Ref. 1). Although initially identified as an anticancer agent (2), TNF has now been shown to be involved in cellular transformation (3), tumor promotion (4), and induction of metastasis (5–7). In agreement with these observations,

mice deficient in TNF have been shown to be resistant to skin carcinogenesis (8). For several tumors, TNF has been shown to be a growth factor (9, 10). Like phorbol ester, TNF mediates these effects in part through activation of a protein kinase C pathway (11). Similar to TNF, other inflammatory cytokines have also been implicated in tumorigenesis (12, 13). Thus, agents that can suppress the expression of TNF and other inflammatory agents have chemopreventive potential (14, 15). Most carcinogens, inflammatory agents, and tumor promoters including cigarette smoke, phorbol ester, okadaic acid, 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, RelA (p65), RelB, NF- κ B1 (p50 and p105), and NF- κ B2 (p52) (16). The NF- κ B proteins are regulated by inhibitors of the I κ B family, which includes I κ B α , I κ B β , I κ B ϵ , I κ B γ , Bcl-3, p100, and p105 (16). 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 IKK. IKK consists of three subunits, IKK- α , IKK- β , and IKK- γ (also called NEMO; for references, see Ref. 17). Gene deletion studies have indicated that IKK- β is essential for NF- κ B activation by most agents (17). The kinase that induces the phosphorylation of p65 is controversial, but IKK- β , protein kinase C, and protein kinase A have been implicated (17–19).

NF- κ B has been shown to regulate the expression of a number of genes whose products are involved in tumorigenesis (20, 21). These include antiapoptosis genes (*e.g.*, cIAP, survivin, TRAF, bcl-2, and bcl-xl); COX-2; MMP-9; genes encoding adhesion molecules, chemokines, inflammatory cytokines, and iNOS; and cell cycle-regulatory genes (*e.g.*, cyclin D1; Ref. 22). Thus, agents that can suppress NF- κ B activation have the potential to suppress carcinogenesis and have therapeutic potential (21, 23). The therapeutic role of phytochemicals in prevention and treatment of cancer has been indicated (24–26). Thus, we sought plant-derived phytochemicals that could suppress NF- κ B activation by various carcinogens.

Ursolic acid (3 β -hydroxy-urs-12-en-28-oic acid) is a pentacyclic triterpenoid (a member of the cyclosqualenoid family; see Fig. 1) derived from berries, leaves, flowers, and fruits of medicinal plants, such as *Rosemarinus officinalis*, *Eriobotrya japonica*, *Calluna vulgaris*, *Ocimum sanctum*, and *Eugenia jambolana* (27). Ursolic acid has been shown to suppress tumorigenesis (28), inhibit tumor promotion (29–31), and suppress angiogenesis (32). Several of these effects of ursolic acid are mediated through suppression of the expression of lipoyxygenase, COX-2, MMP-9, and iNOS (33–39), all of which are genes regulated by NF- κ B. In addition, ursolic acid and its derivatives have been shown to induce apoptosis in a wide variety of cancer cells

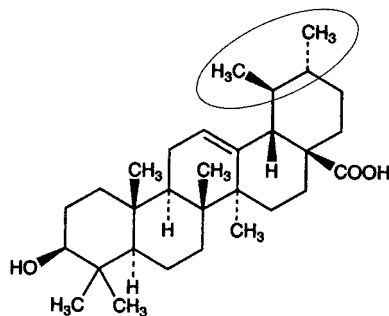
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³ The abbreviations used are: TNF, tumor necrosis factor; TNFR, TNF receptor; NF- κ B, nuclear factor- κ B; SEAP, secretory alkaline phosphatase; IKK, I κ B α kinase; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase 2; MMP-9, matrix metalloproteinase 9; TRADD, TNFR-associated death domain; TRAF2, TNFR-associated factor; NIK, NF- κ B-inducing kinase; PMA, phorbol myristate acetate; EMSA, electrophoretic mobility shift assay.



Ursolic acid

(3 β -hydroxy-urs-12-en-28-oic acid)

Fig. 1. Structure of ursolic acid [(3 β)-3-hydroxyurs-12-en-28-oic acid; C₃₀H₄₈O₅; molecular weight 456.71].

including breast carcinoma, melanoma, hepatoma, prostate carcinoma, and acute myelogenous leukemia (40–46) through inhibition of DNA replication (47), activation of caspases (42, 44, 46), inhibition of protein tyrosine kinases (43), and induction of Ca²⁺ release (48, 49). Another mechanism by which ursolic acid induces apoptosis involves down-regulation of the cellular inhibitor of apoptosis gene (42), another gene known to be regulated by NF- κ B.

Its antitumorigenic, antiangiogenic, and proapoptotic effects, combined with its ability to suppress the expression of lipoxigenase, COX-2, MMP-9, iNOS, and the cellular inhibitor of apoptosis gene, suggest that ursolic acid mediates its effects through suppression of NF- κ B.

Therefore, we investigated in detail the effect of ursolic acid on NF- κ B activation induced by different carcinogens in different cell types. We also investigated the effect of ursolic acid on various steps in the TNF-induced NF- κ B activation pathway, which has been well characterized. The results presented here show that ursolic acid inhibits activation of NF- κ B by several carcinogens through suppression of DNA binding of NF- κ B, I κ B α degradation, I κ B α phosphorylation, I κ B α kinase activation, p65 phosphorylation, p65 nuclear translocation, and NF- κ B-dependent reporter gene expression.

MATERIALS AND METHODS

Materials. Ursolic acid was purchased from Sigma-Aldrich Co. (St. Louis, MO). It was dissolved in ethanol as a 10 mM stock solution and stored at 4°C. Bacteria-derived human recombinant TNF, purified to homogeneity with a specific activity of 5×10^7 units/mg, was kindly provided by Genentech, Inc. (South San Francisco, CA). Penicillin, streptomycin, Iscove's modified Dulbecco's medium, RPMI 1640, and fetal bovine serum were obtained from Life Technologies, Inc. (Grand Island, NY). Tris, glycine, NaCl, SDS, BSA, and PMA were obtained from Sigma Chemical Co. The following polyclonal antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA): anti-p65 (against the epitope corresponding to amino acids mapping within the NH₂-terminal domain of human NF- κ B p65); anti-p50 (against a peptide 15 amino acids long mapping at the nuclear localization sequence region of NF- κ B p50); anti-I κ B α (against amino acids 297–317 mapping at the COOH terminus of I κ B α /MAD-3); and anti-c-Rel and anti-cyclin D1 (against amino acids 1–295, which represents full-length cyclin D1 of human origin). Phospho-I κ B α (Ser³²) antibody was purchased from New England BioLabs (Beverly, MA). Anti-IKK- α and anti-IKK- β antibodies were kindly provided by Imgenex (San Diego, CA). Polyclonal antibody recognizing phosphorylated p65 was obtained from Rockland Laboratories (Gilbertsville, PA). Anti-COX-2 antibody was purchased from Transduction Laboratories (now Invitrogen, Carlsbad, CA), and anti-MMP9 antibody was purchased from Cell Sciences, Inc. (Norwood, MA).

Cell Lines. For most experiments, we used the leukemic cell line Jurkat, which is T cell leukemia. The other cell lines used in this study were 293 (human embryonic kidney), KBM-5 (human myelogenous leukemia), H1299 (human non-small cell lung carcinoma), and U937 (human histiocytic lymphoma); they were obtained from American Type Culture Collection (Manassas, VA). 293 cells were maintained in MEM, KBM-5 cells were maintained in Iscove's modified Dulbecco's medium, and the other cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin.

NF- κ B Activation. To determine NF- κ B activation, we carried out EMSA essentially as described previously (50). Briefly, nuclear extracts prepared from cells (2×10^6 cells/ml) treated with carcinogens were incubated with ³²P-end-labeled 45-mer double-stranded NF- κ B oligonucleotide (4 μ g of protein with 16 fmol of DNA) from the human immunodeficiency virus long terminal repeat, 5'-TTGTTACAAGGGACTTTCCGCTGGGGACTTTCAGGGAGGCGTGG-3' (underline indicates NF- κ B binding sites), for 15 min at 37°C, and the DNA-protein complex formed was separated from free oligonucleotide on 6.6% native polyacrylamide gels. A double-stranded mutated oligonucleotide, 5'-TTGTTACAACCTCACTTTCGCTGCTCACTTTCAGGGAGG CGTGG-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 antibodies against either p50 or p65 of NF- κ B for 30 min at room temperature before the complex was analyzed by EMSA. Antibodies against cyclin D1 and preimmune serum were included as negative controls. The dried gels were visualized, and radioactive bands were quantitated using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) using Imagequant software.

I κ B α Degradation. To determine the effect of ursolic acid on TNF-dependent I κ B α degradation, cytoplasmic extracts were prepared as described previously (51) from Jurkat cells (2×10^6 cells/ml) pretreated with ursolic acid for 8 h and then exposed to 0.1 nM TNF for various times. The extracts were then resolved on 10% SDS-polyacrylamide gels. After electrophoresis, the proteins were electrotransferred to nitrocellulose filters, probed with rabbit polyclonal antibodies against I κ B α , and detected by chemiluminescence (ECL; Amersham). The bands obtained were quantitated using Personal Densitometer Scan v1.30 using Imagequant software version 3.3 (Molecular Dynamics).

I κ B α Phosphorylation. To determine the effect of ursolic acid on TNF-dependent I κ B α phosphorylation, cytoplasmic extracts were prepared from Jurkat cells (2×10^6 cells/ml) treated with 100 μ M ursolic acid for 8 h and then treated with 0.1 nM TNF for various times. The extracts were then resolved on 10% SDS-polyacrylamide gels and analyzed by Western blotting using antibody against phosphorylated I κ B α (Amersham).

IKK Assay. To determine the effect of ursolic acid on TNF-induced IKK activation, we performed IKK assay by a method described previously (52). Briefly, IKK complex was precipitated from whole-cell extracts with antibody to IKK- α and IKK- β followed by treatment with 20 μ l of protein A/G-Sepharose (Pierce, Rockford, IL). After 2 h, 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 of [γ -³²P]ATP, 10 μ M unlabeled ATP, and 2 μ g of substrate glutathione S-transferase-I κ B α (1–54). After incubation at 30°C for 30 min, the reaction was terminated by boiling with 5 μ l of 5 \times SDS sample buffer for 5 min. Finally, the protein was resolved on 10% polyacrylamide gel under reducing conditions, the gel was dried, and the radioactive bands were visualized using a PhosphorImager. To determine the total amounts of IKK- α and IKK- β in each sample, 30 μ g of the whole-cell extract protein were resolved on a 7.5% acrylamide gel and then electrotransferred to a nitrocellulose membrane. The membrane was blocked with 5% nonfat milk protein for 1 h and then incubated with either anti-IKK- α or anti-IKK- β (1:1000 dilution) for 1 h. The membrane was then washed and treated with horseradish peroxidase-conjugated secondary antimouse IgG antibody, and proteins were detected by chemiluminescence (Amersham).

NF- κ B-dependent Reporter Gene Transcription. The effect of ursolic acid on TNF-, TNFR-, TRAF2-, TRADD-, NIK-, and p65-induced NF- κ B-dependent reporter gene transcription was measured as described previously (53). Briefly, human embryonic 293 cells (5×10^5 cells/well) were plated in 6-well plates and transiently transfected the next day 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 18 h. Cells were then treated with 100 μ M ursolic acid for 8 h and then treated with TNF for 24 h. The cell culture medium was then harvested and analyzed for alkaline phosphatase (SEAP) activity essentially according to the protocol described by the manufacturer (Clontech, Palo Alto, CA) using a 96-well fluorescence plate reader (Fluoroscan II; Labsystems, Chicago, IL) with excitation set at 360 nm, and emission set at 460 nm.

NF- κ B p65 Localization. The effect of ursolic acid on TNF-induced nuclear translocation of p65 was examined using an immunocytochemical method as described previously (54). Briefly, treated cells were plated on a poly-L-lysine-coated glass slide by centrifugation using a Cytospin 4 (Thermoshendon, Pittsburgh, PA), air dried for 1 h at room temperature, and fixed with cold acetone. After a brief washing in PBS, slides were blocked with 5% normal goat serum for 1 h and then incubated with rabbit polyclonal antihuman p65 antibody (dilution, 1:100). After overnight incubation, the slides were washed and then incubated with goat antirabbit IgG-Alexa 594 (1:100) for 1 h and counterstained for nuclei with Hoechst (50 ng/ml) for 5 min. Stained slides were mounted with mounting medium (Sigma Chemical Co.) and analyzed under an epifluorescence microscope (Labophot-2; Nikon, Tokyo, Japan). Pictures were captured using a Photometrics Coolsnap CF color camera (Nikon, Lewisville, TX) and MetaMorph version 4.6.5 software (Universal Imaging Corp., Downingtown, PA).

RESULTS

In the present study, we examined the effect of ursolic acid on induction of NF- κ B activation by various carcinogens and inflammatory stimuli and tumor promoters including cigarette smoke, phorbol ester, okadaic acid, H₂O₂, and TNF. The effect of ursolic acid on TNF-induced NF- κ B activation was studied in detail because this pathway has been well characterized. Jurkat cells were used for most

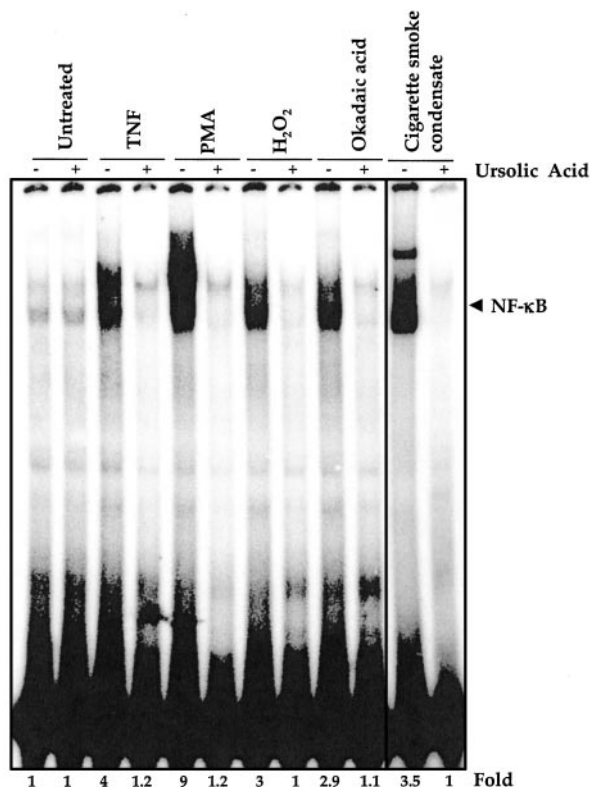


Fig. 2. Ursolic acid blocked induction of NF- κ B activation by TNF, PMA, H₂O₂, okadaic acid, and cigarette smoke condensate. Jurkat cells (2×10^6 cells/ml) were preincubated for 8 h at 37°C with 100 μ M ursolic acid and then treated with TNF (0.1 nM), PMA (100 ng/ml), H₂O₂ (250 μ M), okadaic acid (500 nM), or cigarette smoke condensate (0.1 μ g/ml) for 30 min. Nuclear extracts were prepared and tested for NF- κ B activation as described in "Materials and Methods."

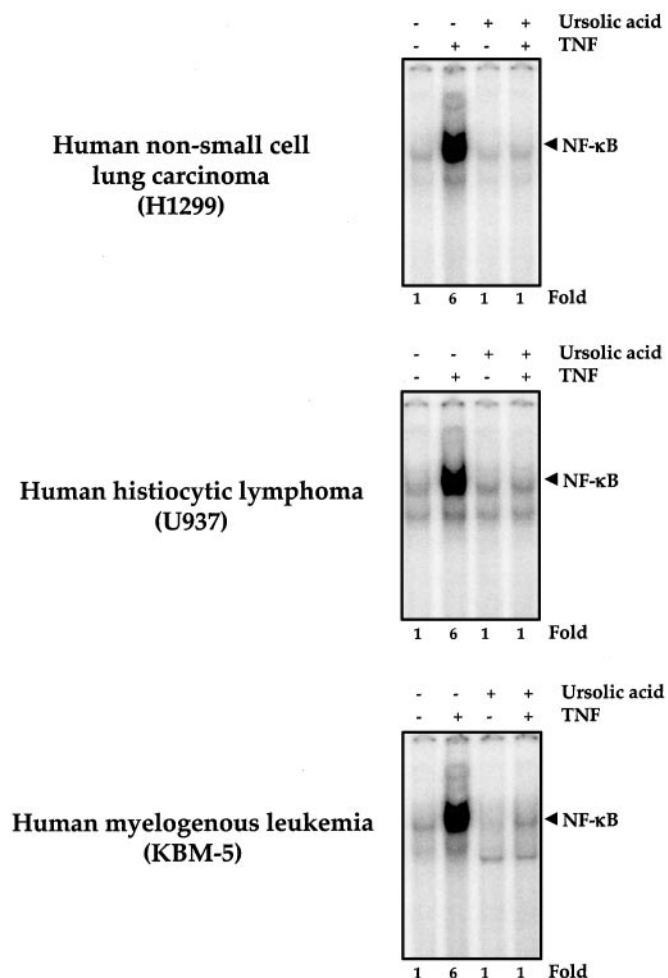


Fig. 3. Ursolic acid inhibited TNF-dependent NF- κ B activation in a variety of cell types. KBM-5, H1299, and U937 cells (2×10^6 cells/ml) were preincubated with or without 100 μ M ursolic acid for 8 h at 37°C and then treated with 0.1 nM TNF for 30 min. Nuclear extracts were prepared and tested for NF- κ B activation as described in "Materials and Methods."

studies because they express both types of TNFRs (TNF- α and TNF- β). The concentration of ursolic acid and NF- κ B activators used and the time of exposure had minimal effect on the viability of these cells as determined by trypan blue dye exclusion test.

Ursolic Acid Blocked Induction of NF- κ B Activation by TNF, PMA, H₂O₂, Okadaic Acid, and Cigarette Smoke. Recent studies from our laboratory (55) have shown that cigarette smoke condensate can activate NF- κ B through phosphorylation and degradation of I κ B α . TNF, PMA, H₂O₂, and okadaic acid are other potent activators of NF- κ B. We therefore examined the effect of ursolic acid on the activation of NF- κ B by these agents in Jurkat human lymphoid cells. As shown in Fig. 2, ursolic acid suppressed induction of NF- κ B activation by all these agents, suggesting that ursolic acid acts at a step in the NF- κ B activation pathway that is common to all these agents.

Inhibition of NF- κ B Activation by Ursolic Acid Was Not Cell Type Specific. It has been demonstrated previously (56) that distinct signal transduction pathways can mediate NF- κ B induction in epithelial and lymphoid cells. We investigated whether ursolic acid could block TNF-induced NF- κ B activation in myeloid cells (KBM-5 and U937) and epithelial cells (H1299). Cells were pretreated with or without ursolic acid and then treated with TNF. Ursolic acid completely inhibited TNF-induced NF- κ B activation in all of the three cell types (Fig. 3), indicating a lack of cell type specificity. Because TNF-induced NF- κ B activation is the best-characterized pathway of

NF- κ B activation, we next investigated the mechanism by which ursolic acid inhibits TNF-induced NF- κ B activation.

Ursolic Acid Inhibits TNF-dependent NF- κ B Activation in a Dose- and Time-dependent Manner. Jurkat cells were preincubated with different concentrations of ursolic acid and then treated with TNF. Nuclear extracts were prepared and tested for NF- κ B activation by EMSA. As shown in Fig. 4A, ursolic acid inhibited TNF-mediated NF- κ B activation in a dose-dependent manner, with maximum inhibition occurring at 100 μ M. Ursolic acid by itself did not activate NF- κ B. Exposure of Jurkat cells to 25, 50, and 100 μ M ursolic acid for 8 h decreased TNF-mediated NF- κ B activation by 0%, 50%, and 100%, respectively, and decreased cell viability by 5%, 9%, and 11%, respectively, suggesting that decrease in NF- κ B activation was not due to loss of cell viability.

We next tested the length of incubation required for ursolic acid to block TNF-induced NF- κ B activation. Cells were preincubated with ursolic acid for different times and then treated with TNF. Complete inhibition of NF- κ B activation was observed at incubation times of 8 h and greater (Fig. 4B).

Because NF- κ B is a family 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 DNA (16). To show that the retarded

band visualized by EMSA in TNF-treated cells was indeed the p50 and p65 subunits of NF- κ B, we incubated nuclear extracts from TNF-activated cells with antibodies to the p50 (NF- κ B1) and the p65 (RelA) subunit of NF- κ B. Both antibodies shifted the band to a higher molecular mass (Fig. 4C), thus suggesting that the TNF-activated complex consisted of p50 and p65 subunits. Neither preimmune serum nor anti-cyclin D1 had any effect. Addition of excess unlabeled NF- κ B (cold oligonucleotide; 100-fold) caused complete disappearance of the band.

It has been shown that the serine protease inhibitor, N- α -tosyl-L-phenylalanyl chloromethyl ketone, herbimycin A, and the protein tyrosine kinase inhibitor caffeic acid phenyl ethyl ester, down-regulate NF- κ B activation by chemically modifying the NF- κ B subunits and thus preventing NF- κ B binding to DNA (57–59). To determine whether ursolic acid also directly modifies NF- κ B proteins, we incubated nuclear extracts from untreated cells and those treated with TNF with various concentrations of ursolic acid for 2 h at room temperature. Then DNA binding activity was detected using EMSA. Our results (Fig. 4D) show that ursolic acid did not modify the DNA binding ability of NF- κ B proteins prepared by treatment with TNF. Therefore, ursolic acid inhibits NF- κ B activation through a mecha-

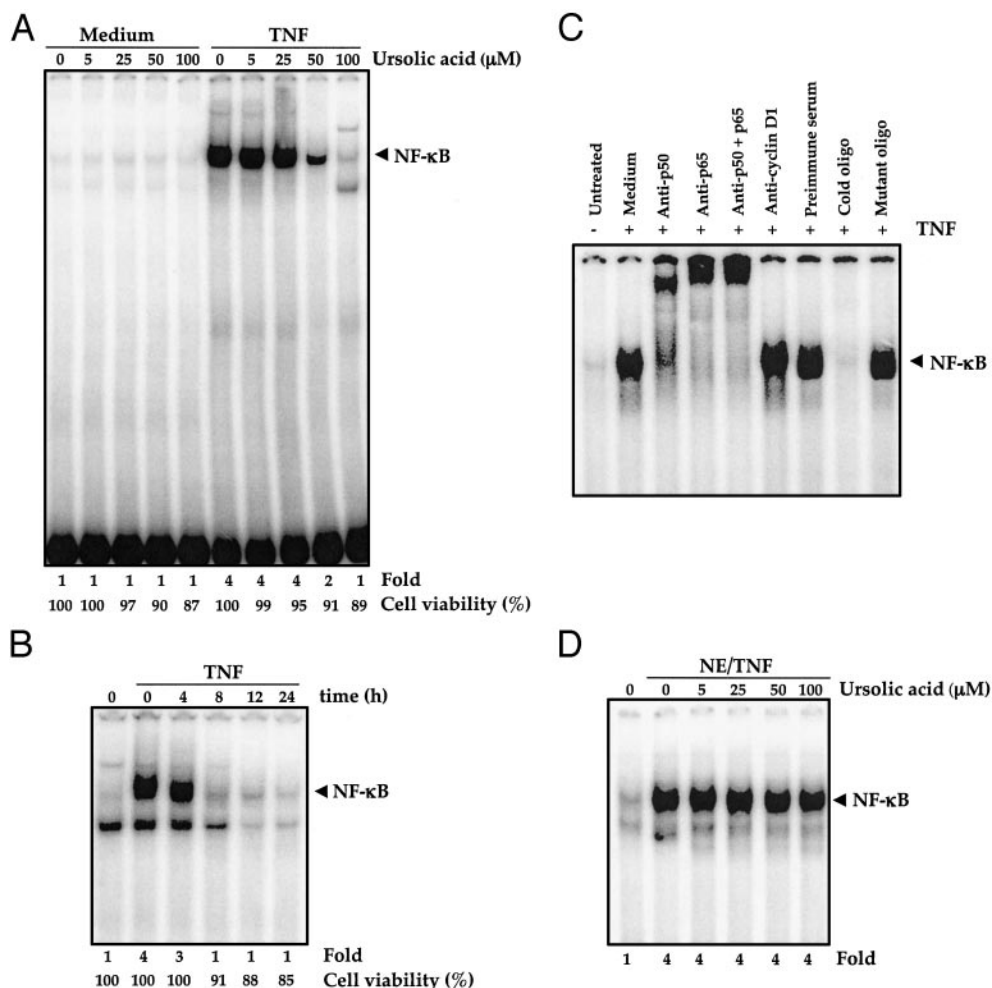


Fig. 4. Ursolic acid inhibited TNF-dependent NF- κ B activation in a dose- and time-dependent manner. *A*, Jurkat cells (2×10^6 cells/ml) were preincubated with different concentrations of ursolic acid for 8 h at 37°C and then treated with 0.1 nM TNF for 30 min. Nuclear extracts were prepared and tested for NF- κ B activation as described in "Materials and Methods." *B*, Jurkat cells (2×10^6 cells/ml) were preincubated with 100 μ M ursolic acid for the indicated times at 37°C and then treated with or without 0.1 nM TNF for 30 min at 37°C. Nuclear extracts were prepared and tested for NF- κ B activation. SDs for percentage of cell viability were less than 5%. *C*, NF- κ B consists of p50 and p65 subunits, and its binding to DNA is specific. Nuclear extracts from Jurkat cells (2×10^6 cells/ml) treated or not treated with 0.1 nM TNF were incubated at 37°C for 30 min with different antibodies or unlabeled NF- κ B oligonucleotide probes and then tested for NF- κ B activation as described in "Materials and Methods." *D*, nuclear extracts from Jurkat cells (2×10^6 cells/ml) treated or not treated with 0.1 nM TNF for 30 min were treated with the indicated concentrations of ursolic acid for 2 h at room temperature and then assayed for DNA binding by EMSA.

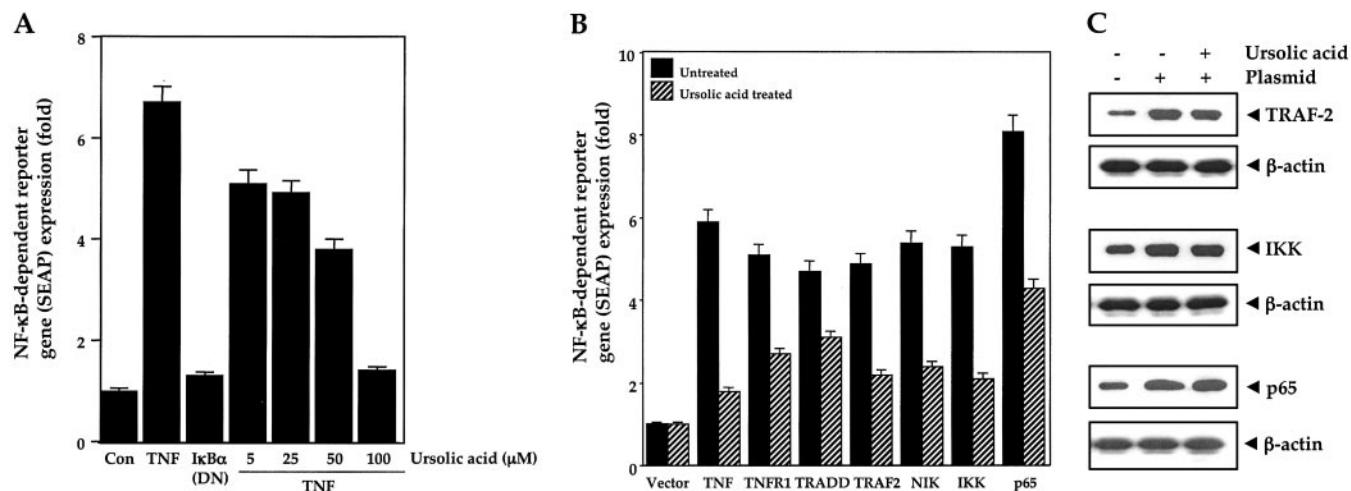


Fig. 5. *A*, ursolic acid inhibited TNF-induced NF- κ B-dependent reporter gene (SEAP) expression. 293 cells treated with the indicated concentrations of ursolic acid were transiently transfected with a NF- κ B-containing plasmid linked to the SEAP gene. After 24 h in culture with 1 nM TNF, cell supernatants were collected and assayed for SEAP activity as described in "Materials and Methods." Results are expressed as fold activity over the activity of the vector control. *B*, ursolic acid inhibited NF- κ B-dependent reporter gene expression induced by TNF, TNFR-1, TRADD, TRAF2, NIK, and IKK. 293 cells were either untreated or treated with 100 μ M ursolic acid for 8 h and then transiently transfected with the indicated plasmids along with a NF- κ B-containing plasmid linked to the SEAP gene. Where indicated, cells were exposed to 1 nM TNF for 12 h. Cell supernatants were assayed for SEAP activity as described in "Materials and Methods." Results are expressed as fold activity over the activity of the vector control. Bars indicate SD. *C*, whole-cell extracts from 293 cells transfected with various plasmids were prepared and analyzed by Western blotting using antibodies against TRAF2, IKK- β , and p65. Equal protein loading was evaluated by β -actin.

nism different from that by which TPCK, herbimycin A, and caffeic acid phenyl ethyl ester inhibit NF- κ B activation.

Ursolic Acid Represses TNF-induced NF- κ B-dependent Reporter Gene Expression. Although we showed by EMSA that ursolic acid blocked NF- κ B activation, DNA binding alone does not always correlate with NF- κ B-dependent gene transcription, suggesting there are additional regulatory steps (60). To determine the effect of ursolic acid on TNF-induced NF- κ B-dependent reporter gene expression, we transiently transfected ursolic acid-pretreated or untreated cells with the NF- κ B-regulated SEAP reporter construct and then stimulated the cells with TNF. An almost 6-fold increase in SEAP activity over the vector control was noted after stimulation with TNF (Fig. 5A). Most of the TNF-induced SEAP activity was abolished by dominant-negative I κ B α , indicating specificity. When the cells were pretreated with ursolic acid, TNF-induced NF- κ B-dependent SEAP expression was inhibited (by 89%) at an ursolic acid concentration of 100 μ M. These results demonstrate that ursolic acid inhibits NF- κ B-dependent reporter gene expression induced by TNF.

TNF-induced NF- κ B activation is mediated through sequential interaction of the TNFR with TRADD, TRAF2, NIK, and IKK- β , resulting in phosphorylation of I κ B α (61, 62). To delineate the site of action of ursolic acid in the TNF-signaling pathway leading to NF- κ B activation, cells were transfected with TNFR1, TRADD, TRAF2, NIK, IKK, and p65 plasmids, and then NF- κ B-dependent SEAP expression was monitored in untreated and ursolic acid-treated cells. As shown in Fig. 5B, TNFR1, TRADD, TRAF2, NIK, IKK, and p65 plasmids induced gene expression, and ursolic acid suppressed reporter gene expression induced by these plasmids. Because phosphorylation of I κ B α and p65 is needed for NF- κ B activation (17), it is possible that ursolic acid inhibits the kinase involved in their phosphorylation. To verify that the expression vectors used indeed produce the predicted protein in the transfected cells, whole-cell extracts were prepared and analyzed by Western blot using antibodies against TRAF2, IKK- β , and p65 (Fig. 5C). It was observed that these plasmids indeed enhanced the expression of the predicted proteins over the basal levels. Ursolic acid treatment, however, did not alter the levels of the protein expression.

Ursolic Acid 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 (16). To determine whether inhibition of TNF-induced NF- κ B activation was due to inhibition of I κ B α degradation, we pretreated cells with ursolic acid and then exposed them to TNF for different time periods. We then examined the cells for NF- κ B in the nucleus by EMSA and

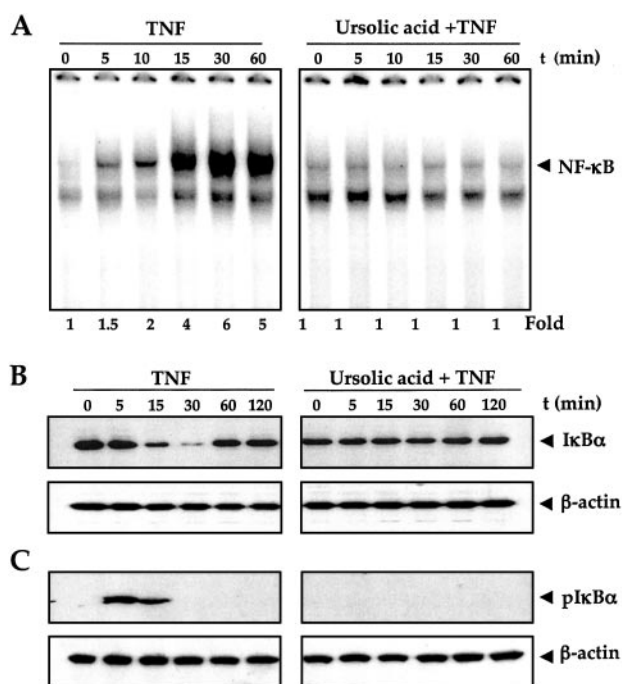


Fig. 6. Ursolic acid inhibited TNF-induced NF- κ B activation, I κ B α degradation, and I κ B α phosphorylation. Jurkat cells (2×10^6 cells/ml) were incubated with 100 μ M ursolic acid for 8 h at 37°C and then treated with 0.1 nM TNF for different times as indicated at 37°C and tested for NF- κ B activation by EMSA (*A*), for I κ B α in cytosolic fractions by Western blot analysis (*B*), and for phosphorylated I κ B α by Western blot analysis with antibodies against phosphorylated I κ B α (*C*). Equal protein loading was evaluated by β -actin.

for I κ B α in the cytoplasm by Western blot analysis. As shown in Fig. 6A, TNF activated NF- κ B in the control cells in a time-dependent manner but had little effect on ursolic acid-pretreated cells. TNF induced I κ B α degradation in control cells as early as 15 min, but in ursolic acid-pretreated cells, TNF had no effect on I κ B α degradation (Fig. 6B). These results indicate that ursolic acid inhibited both TNF-induced NF- κ B activation and I κ B α degradation.

To determine whether ursolic acid affected TNF-induced I κ B α phosphorylation, we examined the TNF-induced phosphorylated form of I κ B α by Western blot analysis, using antibody that detects only the serine-phosphorylated form of I κ B α . TNF induced I κ B α phosphorylation as early as 5 min, and ursolic acid almost completely suppressed the I κ B α phosphorylation (Fig. 6C).

Ursolic Acid Inhibits TNF-induced Nuclear Translocation of p65. TNF has been shown to induce the nuclear translocation of the p65 subunit after phosphorylation. Therefore, we also tested the effect of ursolic acid on TNF-induced nuclear translocation of p65 by Western blot analysis (Fig. 7A) and immunocytochemistry (Fig. 7B). TNF induced the nuclear translocation of p65, and ursolic acid treatment abrogated the p65 translocation.

Ursolic Acid Inhibits TNF-induced Phosphorylation of p65. TNF has been shown to induce the phosphorylation of p65, which is required for translocation of p65 to the nucleus. Therefore, we also tested the effect of ursolic acid on TNF-induced phosphorylation of p65. As shown in Fig. 8A, TNF induced the phosphorylation of p65 in a time-dependent manner, and ursolic acid treatment suppressed p65 phosphorylation almost completely.

Ursolic Acid Inhibits TNF-induced IKK Activation. It has been shown that IKK is required not only for TNF-induced phosphorylation of I κ B α but also for phosphorylation of p65 (19). Because ursolic acid inhibits the phosphorylation of both I κ B α and p65, we tested the effect of ursolic acid on TNF-induced IKK activation. As shown in Fig. 8B, in immune complex kinase assays, TNF activated IKK, and the activation could be seen as early as 5 min after TNF treatment (*top panel*). Ursolic acid treatment completely suppressed the TNF-induced activation of IKK. TNF or ursolic acid had no direct effect on the expression of either IKK- α (Fig. 8B, *middle panel*) or IKK- β (Fig. 8B, *bottom panel*).

To evaluate whether ursolic acid binds directly with IKK proteins,

we incubated whole-cell extracts from untreated cells and cells treated with TNF with various concentrations of ursolic acid. Then kinase assay was performed. The results (Fig. 8C) showed that ursolic acid does not directly bind with IKK but inhibits it by an indirect mechanism.

Ursolic Acid Inhibits TNF-induced COX-2, MMP-9, and Cyclin D1 Activation. Our results indicated that TNF activates NF- κ B through activation of IKK, leading to phosphorylation and degradation of I κ B α , and that NF- κ B is transcriptionally active. TNF treatment induces COX-2 and MMP-9, which are NF- κ B-regulated genes (20–22, 63). We examined whether ursolic acid inhibits induction of COX-2 and MMP-9 by TNF. Jurkat cells were pretreated with ursolic acid and then treated with TNF for different times. Whole-cell extracts were prepared and analyzed by Western blotting for the expression of COX-2 and MMP-9 (Fig. 9A). TNF induced both COX-2 and MMP-9 in a time-dependent manner, and ursolic acid blocked TNF-induced expression of COX-2 and MMP-9. Ursolic acid also blocked the expression of cyclin D1 in TNF-treated and cigarette smoke condensate-treated H1299 cells (Fig. 9B). These results further strengthen the role of ursolic acid in blocking TNF-induced NF- κ B activation.

DISCUSSION

Ursolic acid is an anti-inflammatory, immunomodulatory, and antiproliferative triterpene that has been shown to interfere with the cytokine signaling pathway. NF- κ B plays a central role in the effects attributed to ursolic acid. Therefore, we postulated that ursolic acid must suppress NF- κ B activation. Our results indeed demonstrate that ursolic acid can suppress NF- κ B activation induced by various carcinogens, inflammatory agents, and tumor promoters. These effects were not cell type specific. Ursolic acid inhibited DNA binding of NF- κ B consisting of p50 and p65 and inhibited I κ B α degradation, I κ B α phosphorylation, IKK activation, p65 phosphorylation, p65 nuclear translocation, and NF- κ B-dependent reporter gene expression. Ursolic acid also inhibited NF- κ B-dependent reporter gene expression activated by TNFR, TRADD, TRAF2, NIK, IKK, and p65. The inhibition of NF- κ B activation correlated with suppression of NF- κ B-dependent cyclin D1, COX-2, and MMP-9 expression.

We showed that activation of NF- κ B by TNF, phorbol ester,

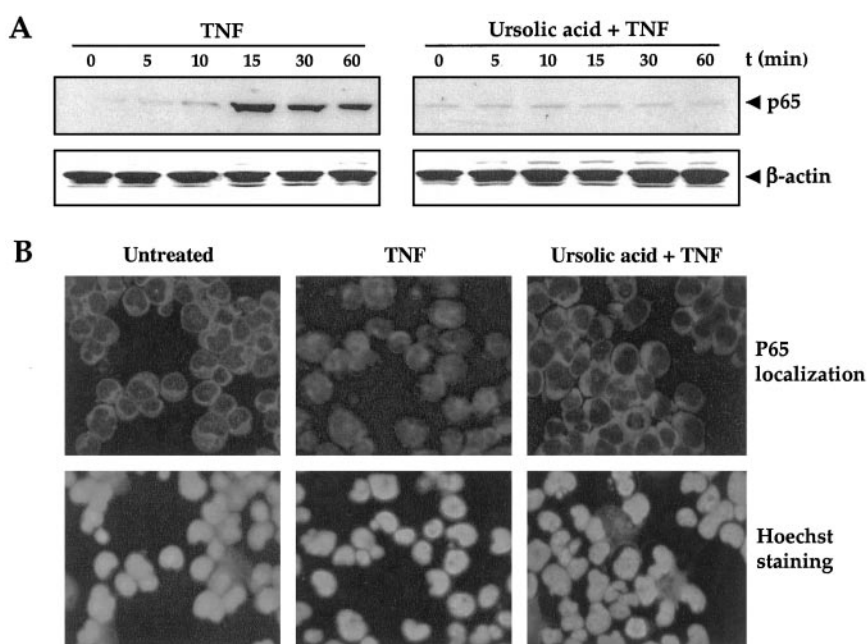


Fig. 7. A, ursolic acid inhibited TNF-induced nuclear translocation of p65. Jurkat cells (1×10^6 cells/ml) were either untreated or pretreated with $100 \mu\text{M}$ ursolic acid for 8 h at 37°C and then treated with 0.1 nM TNF for different times. A, nuclear extracts were prepared and analyzed by Western blotting using antibodies against p65. B, ursolic acid inhibits TNF-induced nuclear translocation of p65. Jurkat cells (1×10^6 cells/ml) were either untreated or pretreated with $100 \mu\text{M}$ ursolic acid for 8 h at 37°C and then treated with 1 nM TNF. After cytospin, immunocytochemical analysis was performed as described in "Materials and Methods."

okadaic acid, H₂O₂, and cigarette smoke was blocked by ursolic acid. These findings suggested that they shared a common pathway. Our results are consistent with a report by Suh *et al.* (39), who showed that induction of NF- κ B in primary mouse macrophages by IFN- γ , lipopolysaccharide, or TNF was inhibited by TP-72, a highly potent analogue of ursolic acid. A concentration of 20 μ M was required to inhibit lipopolysaccharide + IFN- γ -induced NF- κ B activation by 50%. In our studies, 50 μ M ursolic acid suppressed TNF-induced NF- κ B activation by 50% (see Fig. 4). Suh *et al.* (39) did not report how TP-72 suppresses NF- κ B activation. Our studies, however, indicate that ursolic acid inhibits IKK, leading to inhibition of I κ B α phosphorylation and degradation and suppression of p65 nuclear translocation and ultimately resulting in abrogation of DNA binding of NF- κ B and gene transcription. We showed that ursolic acid does not interfere with the binding of NF- κ B to the consensus DNA binding sites. Interestingly, ursolic acid alone at 5 and 10 μ M has been reported to enhance the NF- κ B activation in resting murine macrophages (64). We tested lymphoid, myeloid, and epithelial cells of human origin. We found that up to 100 μ M ursolic acid alone had no

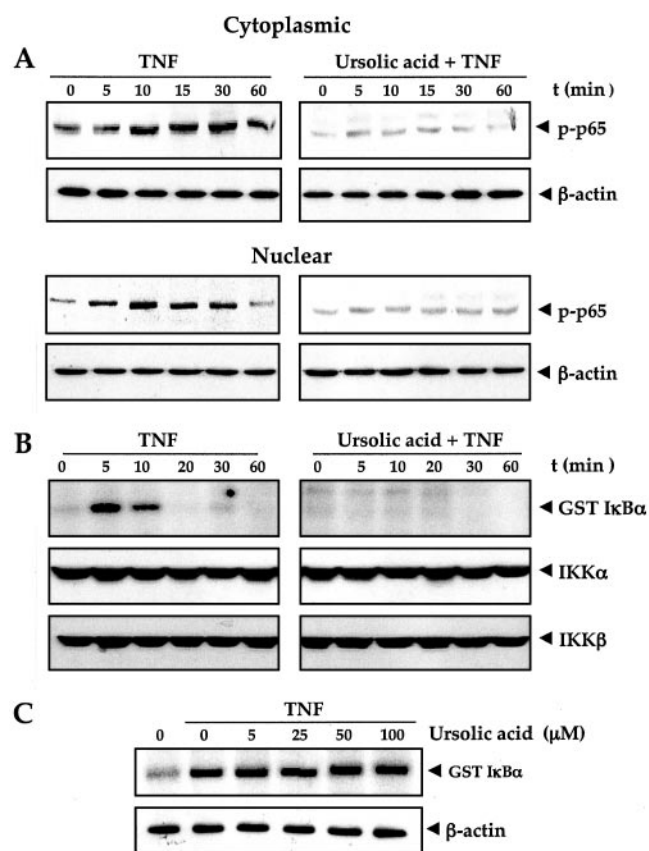


Fig. 8. A, ursolic acid inhibited TNF-induced phosphorylation of p65. Jurkat cells (2×10^6 cells/ml) were incubated with 100 μ M ursolic acid for 8 h and then treated with 0.1 nM TNF for different times. The nuclear and cytoplasmic extracts were analyzed by Western blotting using antibodies against the phosphorylated form of p65. B, ursolic acid inhibited TNF-induced IKK activity. Jurkat cells (2×10^6 cells/ml) were treated with 100 μ M ursolic acid for 8 h and then treated with 0.1 nM TNF for different time intervals. Whole-cell extracts were prepared, and 200 μ g of extract were immunoprecipitated with antibodies against IKK- α and IKK- β . Thereafter, immune complex kinase assay was performed as described in "Materials and Methods." To examine the effect of ursolic acid on the level of expression of IKK proteins, 30 μ g of cytoplasmic extracts were run on 7.5% SDS-PAGE, electrotransferred, and immunoblotted with the indicated antibodies. C, ursolic acid did not directly interact with IKK. Whole-cell extracts were prepared from untreated and TNF (0.1 nM)-treated Jurkat cells (2×10^6 cells/ml); 200 μ g/sample whole-cell extract protein was immunoprecipitated with antibodies against IKK- α and IKK- β . The immune complex was treated with the indicated concentrations of ursolic acid for 30 min at 30°C, and then an immune complex kinase assay was performed as described in "Materials and Methods." Equal protein loading was evaluated by β -actin.

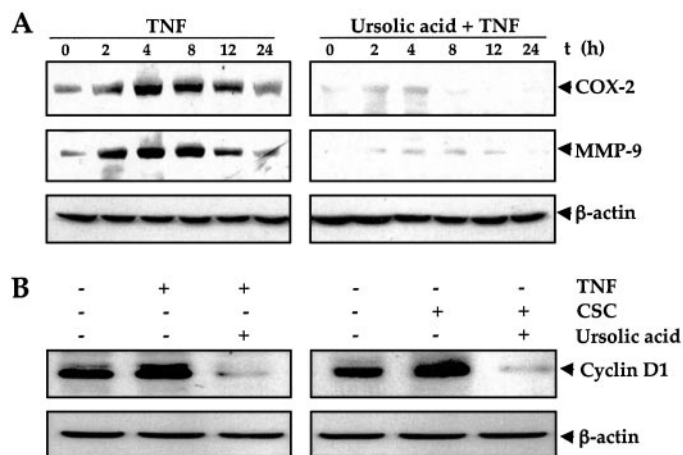


Fig. 9. A, ursolic acid inhibited induction of COX-2 and MMP-9 by TNF. Jurkat cells (2×10^6 cells/ml) were left untreated or incubated with 100 μ M ursolic acid for 8 h and then treated with 0.1 nM TNF for different time periods. Whole-cell extracts were prepared and analyzed by Western blotting using antibodies against COX-2 and MMP-9. B, ursolic acid inhibited TNF-induced cyclin D1. H1299 cells (2×10^6 cells/ml) were left untreated or incubated with 100 μ M ursolic acid for 8 h and then treated with 0.1 nM TNF for different time periods. Whole-cell extracts were prepared, and 80 μ g of the whole-cell lysate were analyzed by Western blotting using antibodies against cyclin D1. CSC, cigarette smoke condensate.

effect on NF- κ B activation. Whether these differences are related to species or cell line is unclear at present.

We found that ursolic acid inhibited TNF-induced activation of IKK. By using antibodies that specifically detect the phosphorylated form of I κ B α , we showed that ursolic acid blocks TNF-induced phosphorylation of I κ B α . The phosphorylation of I κ B α is regulated by a large number of kinases including IKK- α , IKK- β , IKK- γ , NIK, TAK1, Akt, and mitogen-activated protein kinase kinase 1 (17, 21). Akt and NIK are primarily known to activate IKK- α , whereas mitogen-activated protein kinase kinase 1 and atypical protein kinase C activate IKK- β . Ursolic acid inhibited IKK activity without directly interfering with the IKK protein. Thus, it is possible that ursolic acid blocked IKK activation by inhibiting one or many of the upstream kinases responsible for IKK activation.

It is known that TRAF2 binds to TNFR through TRADD and is required for NF- κ B, activator protein 1, and c-Jun-NH₂-terminal kinase activation (61). Ursolic acid inhibited the NF- κ B-dependent reporter gene expression activated by TNFR, TRADD, TRAF2, NIK, IKK, and p65. How ursolic acid suppresses the NF- κ B activation induced by all these signaling intermediates is not clear. Gene deletion studies have shown that TRAF2 and NIK are not involved in TNF-induced NF- κ B activation (17). Several protein kinases have been implicated in TNF-induced NF- κ B activation (17–19). We found that ursolic acid inhibits TNF-induced phosphorylation of the p65 subunit of NF- κ B. Numerous protein kinases are known to regulate the phosphorylation of p65. Recent studies have shown that IKK could also phosphorylate p65 (19). This further strengthens the view that ursolic acid blocks IKK activity that phosphorylates I κ B α and p65. Recently, it has been shown that the phosphorylation and acetylation of p65 play a major role in DNA binding and transactivation of NF- κ B (65–67). Mesalamine has been shown to block interleukin 1-induced NF- κ B-dependent reporter activity through suppression of p65 phosphorylation (65). How ursolic acid inhibits p65 phosphorylation is not clear. Because IKK has been shown to phosphorylate p65 (19, 67), it is possible that ursolic acid inhibits p65 phosphorylation through inhibition of IKK. IKK activation was indeed inhibited by ursolic acid in our studies. Ghosh and Karin (17) showed that cyclic AMP-dependent protein kinase A is involved in the phosphorylation

of p65. Thus, ursolic acid could suppress NF- κ B action through inhibition of protein kinase A. Indeed, a previous report indicated that ursolic acid can suppress protein kinase A (68). Thus, it is possible that ursolic acid is inhibiting NF- κ B activation by inhibiting multiple kinases.

How ursolic acid suppresses the activation of IKK is not clear. The role of lipid peroxidation in NF- κ B activation has been implicated (69). We showed previously (70) that TNF can induce lipid peroxidation. It is possible that ursolic acid suppresses NF- κ B activation through suppression of lipid peroxidation. Previously, ursolic acid has been shown to inhibit lipid peroxidation (71).

We found in the present study that ursolic acid suppressed TNF-induced expression of cyclin D1, COX-2, and MMP-9 expression, which are NF- κ B-regulated genes involved in initiation, promotion, and metastasis of tumors (20–22). A number of COX-2 inhibitors possess both anti-inflammatory and chemopreventive properties (72–74). Our results are consistent with other reports demonstrating that ursolic acid blocks expression of COX-2 (34–36, 39) and MMP-9 (37, 38). Ursolic acid is also known to inhibit expression of iNOS (39), another gene known to be regulated by NF- κ B (75). Thus, suppression of iNOS by ursolic acid could also be due to inhibition of NF- κ B activation. The inhibition of expression of these NF- κ B-regulated genes may explain the antitumor effects assigned to ursolic acid (28–31). NF- κ B has also been implicated in suppression of apoptosis (22). Cellular inhibitor of apoptosis is one of the genes that can suppress apoptosis, and it is regulated by NF- κ B. The down-regulation of this gene by ursolic acid (42) could also be due to suppression of NF- κ B. Ursolic acid has also been shown to suppress HIV replication (76), which may also be mediated through inhibition of NF- κ B. Thus, overall, our results suggest that anticarcinogenic, anti-inflammatory, and proapoptotic effects of ursolic acid may be mediated through inhibition of the ability of a wide variety of carcinogens and inflammatory agents to activate NF- κ B.

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