Withanolides potentiate apoptosis, inhibit invasion, and abolish osteoclastogenesis through suppression of nuclear factor-κB (NF-κB) activation and NF-κB–regulated gene expression

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Abstract

The plant *Withania somnifera Dunal* (Ashwagandha), also known as Indian ginseng, is widely used in the Ayurvedic system of medicine to treat tumors, inflammation, arthritis, asthma, and hypertension. Chemical investigation of the roots and leaves of this plant has yielded bioactive withanolides. Earlier studies showed that withanolides inhibit cyclooxygenase enzymes, lipid peroxidation, and proliferation of tumor cells. Because several genes that regulate cellular proliferation, carcinogenesis, metastasis, and inflammation are regulated by activation of nuclear factor-κB (NF-κB), we hypothesized that the activity of withanolides is mediated through modulation of NF-κB activation. For this report, we investigated the effect of the withanolide on NF-κB and NF-κB–regulated gene expression activated by various carcinogens. We found that withanolides suppressed NF-κB activation induced by a variety of inflammatory and carcinogenic agents, including tumor necrosis factor (TNF), interleukin-1β, doxorubicin, and cigarette smoke condensate. Suppression was not cell type specific, as both inducible and constitutive NF-κB activation was blocked by withanolides. The suppression occurred through the inhibition of inhibitory subunit of IκBα kinase activation, IκBα phosphorylation, IκBα degradation, p65 phosphorylation, and subsequent p65 nuclear translocation. NF-κB–dependent reporter gene expression activated by TNF, TNF receptor (TNFR) 1, TNFR-associated death domain, TNFR-associated factor 2, and IκBα kinase was also suppressed. Consequently, withanolide suppressed the expression of TNF-induced NF-κB–regulated antiapoptotic (inhibitor of apoptosis protein 1, Bfl-1/A1, and FADD-like interleukin-1β-converting enzyme–inhibitory protein) and metastatic (cyclooxygenase-2 and intercellular adhesion molecule-1) gene products, enhanced the apoptosis induced by TNF and chemotherapeutic agents, and suppressed cellular TNF-induced invasion and receptor activator of NF-κB ligand-induced osteoclastogenesis. Overall, our results indicate that withanolides inhibit activation of NF-κB and NF-κB–regulated gene expression, which may explain the ability of withanolides to enhance apoptosis and inhibit invasion and osteoclastogenesis. [Mol Cancer Ther 2006;5(6):1434–45]

Introduction

Withania somnifera Dunal is one of the most ancient and sought-after herbs for the preparation of herbal formulations and dietary supplements. It belongs to the family Solanaceae and is distributed throughout India. In traditional Indian medicine, or Ayurveda, the leaves and roots were prescribed to cure inflammation-related disorders (1). This plant has been studied extensively for its biologically active constituents and has yielded several steroidal lactones called withanolide (2, 3). A pharmacologic study conducted on this plant indicated that a component withanolide, withaferin A, inhibits angiogenesis (4). Withanolides have also been reported to inhibit metastasis (5) and quinone reductase activity (6). Some of them have been shown to preferentially affect events in the cholinergic signal transduction cascade of the cortical and basal forebrain, indicating their promise for the treatment of Alzheimer's disease (7). Despite these studies of withanolide pharmacologic activity, their mechanisms of action remain unknown.

We postulated, based on similarities to other natural pharmaceuticals that we have studied, that withanolides mediate their effects through suppression of the transcription factor nuclear factor-κB (NF-κB). The evidence

Received 2/21/06; revised 4/1/06; accepted 4/27/06.

Grant support: Clayton Foundation for Research, Department of Defense-U.S. Army Breast Cancer Research Program grant BC010610, NIH Lung Chemoprevention PO1 grant CA91844, and NIH P50 Head and Neck Specialized Programs of Research Excellence grant P50CA97007 (B.B. Aggarwal).

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Note: B.B. Aggarwal is a Ransom Horne, Jr., professor of cancer research.

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doi:10.1158/1535-7163.MCT-06-0096

is multifaceted: (*a*) NF-κB is activated by various carcinogens, tumor promoters, and conditions in the tumor microenvironment (hypoxia and acidic pH); (*b*) most inflammatory agents activate NF-κB; (*c*) NF-κB regulates the expression of genes that regulate transformation, tumor promotion, tumor invasion, angiogenesis, and metastasis; (*d*) suppression of apoptosis is regulated by NF-κB; and (*e*) chemopreventive agents have been shown to suppress NF-κB activation (8).

NF-κB is a heterodimeric protein complex consisting of members of the Rel (p50)/NF-κB (p65) protein family. It is primarily composed of proteins with molecular masses of 50 kDa (p50) and 65 kDa (p65) and is retained in the cytoplasm by its inhibitory subunit, IκBα. In its unstimulated form, NF-κB is activated by a wide variety of inflammatory stimuli, including tumor necrosis factor (TNF), interleukin (IL)-1, phorbol 12-myristate 13-acetate, hydrogen peroxide, endotoxin, and γ-irradiation. Most of these agents induce the phosphorylation-dependent degradation of IκBα proteins, allowing active NF-κB to translocate to the nucleus, where it regulates gene expression. The phosphorylation of IκBα is mediated through the activation of the IκBα kinase (IKK) complex consisting of IKK-α, IKK-β, IKK-γ (also called NEMO), IKK-associated protein 1, FIP-3 (type 2 adenovirus E3-14.7-kDa interacting protein), heat shock protein 90, and glutamic acid, leucine, lysine, and serine–abundant protein (9).

To test our hypothesis, we investigated the effect of withanolides on NF-κB activation induced by carcinogens, tumor promoters, and inflammatory agents. Our results indicate that withanolide are potent suppressors of NF-κB activation induced by various agents and that this suppression is mediated through inhibition of IKK. This mechanism accounts for the ability of withanolides to suppress the expression of gene products that regulate apoptosis, proliferation, angiogenesis, and invasion.

Materials and Methods

Reagents

A 20 mmol/L solution of withanolide was prepared with DMSO, stored as small aliquots at −20°C, and then thawed and diluted as needed in cell culture medium. Bacteria-derived human recombinant TNF, purified to homogeneity with a specific activity of 5×10^7 units/mg, was kindly provided by Genentech (South San Francisco, CA). Cigarette smoke condensate, prepared as described (10), was kindly supplied by Dr. G. Gairola (University of Kentucky, Lexington, KY). Penicillin, streptomycin, RPMI 1640, Iscove's modified Dulbecco's medium, DMEM, DMEM/F-12, and fetal bovine serum were obtained from Invitrogen (Grand Island, NY). Anti-β-actin antibody was obtained from Sigma-Aldrich (St. Louis, MO). The antibodies anti-p65, anti-p50, anti-IκBα, anti–poly(ADP-ribose) polymerase, anti–intercellular adhesion molecule-1, anti–inhibitor of apoptosis protein 1, and anti-Bfl-1/A1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–cyclooxygenase (COX)-2 was obtained from BD Biosciences (San Diego, CA). Phosphospecific anti-IκBα (Ser^{32}) and phosphospecific anti-p65 (Ser^{536}) were purchased from Cell Signaling (Beverly, MA). Anti-IKK-α, anti-IKK-β, and anti–FADD-like IL-1β-converting enzyme–inhibitory protein antibodies were kindly provided by Imgenex (San Diego, CA).

Cell Lines

KBM-5 (human chronic myeloid leukemia), A293 (human embryonic kidney carcinoma), MCF-7 (human breast adenocarcinoma), and RAW 264.7 (murine monocytic cell) cells were obtained from American Type Culture Collection (Manassas, VA). KBM-5 cells were cultured in Iscove's modified Dulbecco's medium with 15% fetal bovine serum. MCF-7 and U266 cells were obtained from American Type Culture Collection. MCF-7 and U266 cells were cultured in RPMI 1640, A293 cells were cultured in DMEM, and RAW 264.7 cells were cultured in DMEM/F-12 supplemented with 10% fetal bovine serum. Culture media were also supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin.

Isolation of Withanolides

The withanolide studied in this report were isolated from *W. somnifera* leaf extract as described earlier (2). The leaf extract was purchased from Phytomyco Research Corp. (Greensville, NC). The extract (5 g) was stirred with *n*-hexane to remove chlorophyll and other lipid-soluble components, and the resulting residue (3.2 g) obtained was fractionated by medium-pressure liquid chromatography using $CHCl_3$ and methanol (v/v) gradients. A total of 120 fractions in 15-mL aliquots were collected. Based on analytic TLC, the fractions were combined to yield fractions I (600 mg), II (500 mg), III (1,200 mg), and IV (200 mg). Compounds WS-1 (120 mg) and WS-3 (50 mg) were obtained by repeated purification of fraction I using column chromatography with hexane/ethyl acetate (1:1, v/v) as the mobile phase. Fraction II was purified by preparative high-performance liquid chromatography (HPLC) using methanol/H_2O (1:1, v/v) as the mobile phase; this step yielded an impure fraction (50 mg), which was further purified by HPLC using methanol/H_2O (4:6, v/v) as the mobile phase to produce WS-6 (40 mg). The purification of fraction III by HPLC (methanol/H_2O; 6:4, v/v) yielded a pure compound, WS-5 (15 mg), and fractions I (950 mg) and II (16 mg). Further purification of fraction I by HPLC (methanol/H_2O; 6:4, v/v) resulted in WS-7 (200 mg). Similarly, purification of fraction II by HPLC (methanol/H_2O; 7:3, v/v) yielded WS-4 (12 mg). Fraction IV was subjected to HPLC purification (methanol/H_2O; 7:3, v/v) to yield fractions III (13 mg) and IV (30 mg). These fractions were further purified by HPLC using methanol/H_2O (6:4, v/v) as the mobile phase to produce the compounds WS-9 (10.5 mg), WS-10 (8.5 mg), and WS-11 (5.0 mg). Compounds WS-1 and WS-7 were acetylated using Ac_2O/pyridine to yield the acetylated derivatives WS-2 and WS-8.

Electrophoretic Mobility Shift Assays

To determine NF-κB activation, we did electrophoretic mobility shift assays (EMSA) as described previously (11). Briefly, nuclear extracts prepared from TNF-treated cells were incubated with ^{32}P-end-labeled 45-mer double-stranded NF-κB oligonucleotide (15 μg protein with 16 fmol DNA) from the HIV long terminal repeat, 5′-TTGTTACAA**GGGACTTTC**CGCTG**GGGACTTTC**-CAGGGAGGCGTGG-3′ (boldface indicates NF-κB binding sites), for 30 minutes 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′-TTGTTACAACTCACTTTCC-GCTGCTCACTTTCCAGGGAGGCGTGG-3′, was used to examine the specificity of binding of NF-κB to the DNA. The specificity of binding was also examined through competition with the unlabeled oligonucleotide. The dried gels were visualized, and radioactive bands were quantitated by a PhosphorImager STORM 220 (Amersham Biosciences, Piscataway, NJ) using ImageQuant (Molecular Dynamics, Sunnyvale, CA) software.

Western Blot Analysis

To determine the levels of protein expression in the cytoplasm or nucleus, we prepared extracts (12) and fractionated them using SDS-PAGE. After electrophoresis, the proteins were electrotransferred to nitrocellulose membranes, blotted with each antibody, and detected by enhanced chemiluminescence reagent (Amersham Biosciences). The bands obtained were quantitated using NIH imaging software (NIH, Bethesda, MD).

IKK Assay

To determine the effect of withanolide on TNF-induced IKK activation, IKK assay was done by a method described previously (13). In brief, to determine the total amounts of IKK-α and IKK-β in each sample, 50 μg whole-cell protein was resolved on 7.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with either anti-IKK-α or anti-IKK-β antibodies.

NF-κB-Dependent Reporter Gene Expression Assay

NF-κB-dependent reporter gene expression assay was done as described (14). The effects of withanolide on TNF-, TNF receptor (TNFR)–, TNFR-associated death domain

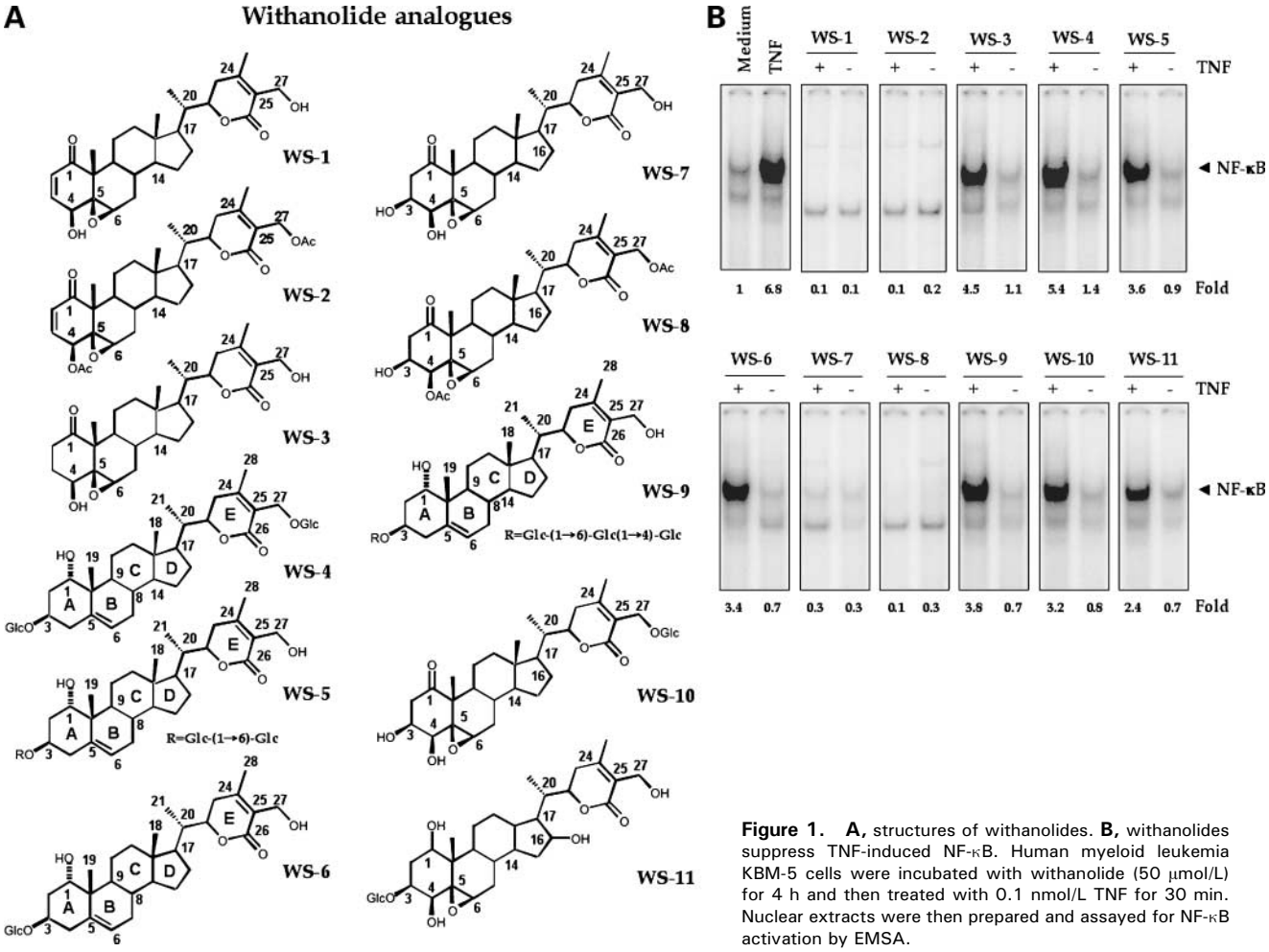

Figure 1. **A,** structures of withanolides. **B,** withanolides suppress TNF-induced NF-κB. Human myeloid leukemia KBM-5 cells were incubated with withanolide (50 μmol/L) for 4 h and then treated with 0.1 nmol/L TNF for 30 min. Nuclear extracts were then prepared and assayed for NF-κB activation by EMSA.

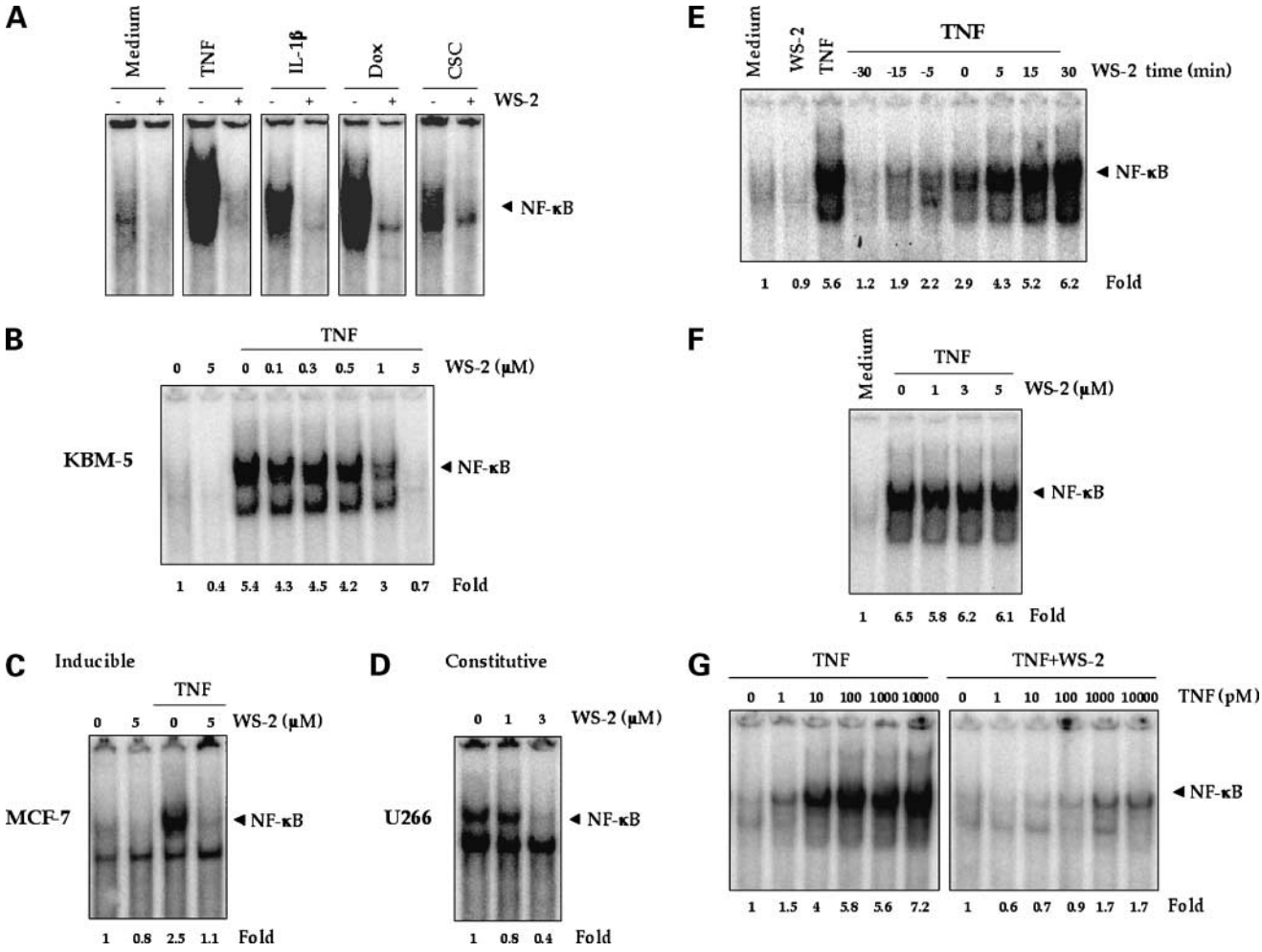

Figure 2. Withanolide suppresses TNF-induced NF-κB in a dose-dependent manner and in different cell lines. **A,** withanolide blocks NF-κB activation induced by TNF, IL-1β, doxorubicin, and cigarette smoke condensate. Human myeloid leukemia KBM-5 cells were preincubated for 30 min at 37°C with 5 μmol/L WS-2 and then treated with TNF (1 nmol/L, 30 min), IL-1β (100 ng/mL, 30 min), doxorubicin (*Dox*; 20 μmol/L, 3 h), and cigarette smoke condensate (*CSC*; 10 μg/mL, 1 h). Nuclear extracts were then prepared and assayed for NF-κB activation using EMSA. **B,** withanolide suppresses TNF-induced NF-κB activation in a dose-dependent manner. KBM-5 cells were preincubated with the indicated concentrations of WS-2 for 30 min and then treated with 0.1 nmol/L TNF for 30 min. Nuclear extracts were then prepared and assayed for NF-κB activation by EMSA. **C,** withanolide blocks TNF-induced NF-κB activation in human breast adenocarcinoma MCF-7 cells preincubated with 5 μmol/L WS-2 for 30 min and 0.1 nmol/L TNF for 30 min. **D,** withanolide suppresses constitutive activation of NF-κB in U266 human multiple myeloma cells. Cells were coincubated with the indicated concentrations of WS-2. Nuclear extracts were then prepared and assayed for NF-κB activation by EMSA. **E,** suppression of NF-κB by withanolide is time dependent. KBM-5 cells were preincubated with 5 μmol/L WS-2 for the indicated times and then tested for NF-κB activation at 37°C for 30 min with 0.1 nmol/L TNF. - and +, WS-2 was added before or after TNF, respectively. After these treatments, nuclear extracts were prepared and assayed for NF-κB activation by EMSA. **F,** direct effect of WS-2 on the NF-κB complex. Nuclear extracts were prepared from untreated or 0.1 nmol/L TNF-treated KBM-5 cells, incubated for 30 min with indicated concentrations of WS-2, and then assayed for NF-κB activation by EMSA. **G,** withanolide is a potent inhibitor of TNF-induced NF-κB activation. KBM-5 cells were preincubated with 5 μmol/L WS-2 for 30 min, incubated with indicated concentrations of TNF for 30 min, and then subjected to EMSA for NF-κB activation.

(TRADD)−, TNFR-associated factor 2 (TRAF2)−, NF-κB-inducing kinase (NIK)−, IKK-β-, and p65-induced NF-κB-dependent reporter gene transcription were analyzed by secretory alkaline phosphatase (SEAP) assay as described previously (14).

Immunocytochemistry for NF-κB p65 Localization

The effect of withanolide on the nuclear translocation of p65 was examined by immunocytochemistry as described previously (12).

Live and Dead Assay

To measure apoptosis, we used the Live and Dead assay (Molecular Probes, Eugene, OR), which determines intracellular esterase activity and plasma membrane integrity. This assay was done as indicated previously (15).

Cytotoxicity Assay

The effect of withanolide on the cytotoxic effects of TNF and chemotherapeutic agents was determined by the

3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide uptake method as described (16).

Terminal Deoxynucleotidyl Transferase–Mediated dUTP Nick End Labeling Assay

We also assayed cytotoxicity using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling method, which examines DNA strand breaks during apoptosis using an *in situ* cell death detection reagent. This assay was done as indicated previously (17).

Invasion Assay

Invasion through the extracellular matrix is a crucial step in tumor metastasis. We used Matrigel basement membrane matrix extracted from the Engelbreth-Holm-Swarm mouse tumor as a reconstituted basement membrane for *in vitro* invasion assays. This assay was done as indicated previously (17).

Osteoclast Differentiation Assay

RAW 264.7 cells were cultured in 24-well dishes at a density of 1×10^4 per well and were allowed to

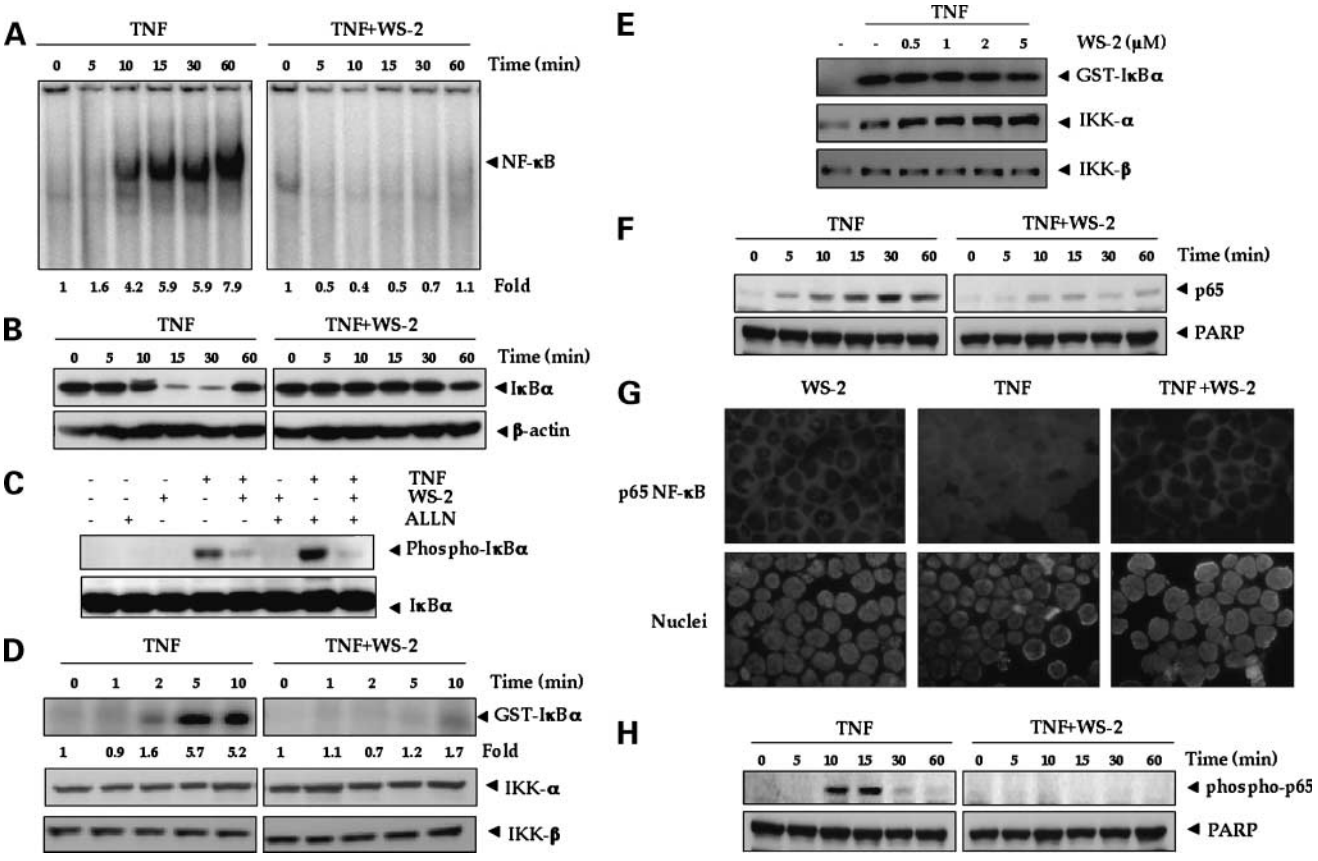

Figure 3. Effect of withanolide on IκBα phosphorylation and degradation induced by TNF. **A,** withanolide inhibits TNF-induced activation of NF-κB in a time-dependent manner. KBM-5 cells were preincubated with 5 μmol/L WS-2 for 30 min and then treated with 0.1 nmol/L TNF for indicated times and analyzed for NF-κB activation by EMSA. **B,** effect of withanolide on TNF-induced degradation of IκBα. KBM-5 cells were preincubated with 5 μmol/L WS-2 and 0.1 nmol/L TNF for the indicated times. Cytoplasmic extracts were prepared, fractionated on 10% SDS-PAGE, and electrotransferred to nitrocellulose membrane. Western blot analysis was done with anti-IκBα. **C,** withanolide suppresses TNF-induced phosphorylation of IκBα. KBM-5 cells were preincubated with 5 μmol/L WS-2 and then treated with 0.1 nmol/L TNF for 15 min after added *N*-acetyl-Leu-Leu-norleucinal (*ALLN*). Cytoplasmic extracts were prepared, fractionated on 10% SDS-PAGE, and electrotransferred to nitrocellulose membrane. Western blot analysis was done with anti-phosphospecific IκBα and anti-IκBα. **D,** withanolide suppresses the TNF-induced activation of IKK. KBM-5 cells were pretreated with 50 ng/mL *N*-acetyl-Leu-Leu-norleucinal for 30 min, preincubated with 5 μmol/L WS-2 for 30 min, and then treated with 1 nmol/L TNF for the indicated times. Whole-cell extracts were immunoprecipitated with antibody against IKK-α and analyzed by immune complex kinase assay as described in Materials and Methods. To examine the effect of withanolide on the level of IKK proteins, whole-cell extracts were fractionated on SDS-PAGE and examined by Western blot analysis using anti-IKK-α and anti-IKK-β antibodies. **E,** direct effect of withanolide on the activation of IKK induced by TNF. Whole-cell extracts were prepared from 1 nmol/L TNF-treated KBM-5 cells and immunoprecipitated with IKK-α antibody. The immune complex kinase assay was then done in the absence or presence of the indicated concentration of WS-2. **F,** effect of withanolide on TNF-induced translocalization of p65. KBM-5 cells were preincubated with 5 μmol/L WS-2, and 0.1 nmol/L TNF was added for the indicated times. Nuclear extracts were prepared, fractionated on 10% SDS-PAGE, and electrotransferred to nitrocellulose membrane. Western blot analysis was done with anti-p65 antibodies. **G,** immunocytochemical analysis of p65 localization. KBM-5 cells were preincubated with 5 μmol/L WS-2 and then 1 nmol/L TNF for 15 min and subjected to immunocytochemical analysis as described in Materials and Methods. **H,** effect of withanolide on TNF-induced phosphorylation of p65. KBM-5 cells were preincubated with 5 μmol/L WS-2 for 30 min and then treated with 0.1 nmol/L TNF for the indicated times. Nuclear extracts were prepared, fractionated on 10% SDS-PAGE, and electrotransferred to nitrocellulose membrane. Western blot analysis was done with phosphospecific p65.

adhere overnight. This assay was done as indicated previously (18).

Results

The aim of the current study was to establish the effects of withanolide on the NF-κB activation pathway induced by various carcinogens and inflammatory stimuli, on NF-κB-regulated gene expression, and on NF-κB-mediated cellular responses. Because the TNF-induced NF-κB activation pathway has been well characterized, we investigated in detail the effects of withanolide on TNF-induced NF-κB activation. The structures of the various withanolides we investigated are shown in Fig. 1A.

Structure and Activity Relationship

Based on their structural similarities, the withanolides tested for the inhibition of NF-κB activation were grouped into withaferin A, viscosalactone B, and physagulin D series. Although the physagulin D class of withanolides did not inhibit NF-κB activation, withaferin A (WS-1) and its acetyl derivative withanolide (WS-2) showed potent inhibition (Fig. 1B). Because dihydrowithaferin A (WS-3) did not inhibit NF-κB activation, it was clear that an unsaturated lactone moiety in ring A of WS-1 was important for biological activity. However, it is interesting to note that activity was regained with the addition of a hydroxyl group at the 3-position to produce viscosalactone B (WS-7), although it lacked the C2-C3 double bond. The acetylation of hydroxyl groups in WS-7 did not affect activity, and the resulting derivative, WS-8, inhibited NF-κB activation about as much as the parent molecule. On the other hand, glycosylation at C-27 (e.g., in WS-10) inactivated the NF-κB-inhibiting activity of withanolide and indicated that large substituents at C-27 would reduce the molecules ability to inhibit NF-κB activation. These observations are in accordance with our earlier study of *in vitro* tumor cell proliferation inhibitory activities of withanolide (19). For all following studies, only the WS-2 was investigated.

Withanolide Inhibits NF-κB Activation Induced by Various Agents

Because TNF, IL-1β, doxorubicin, and cigarette smoke condensate are potent activators of NF-κB (10, 16, 20, 21), we examined the effect of WS-2 on the activation of NF-κB by these agents. Incubation of cells with 5 μmol/L WS-2 suppressed the activation of NF-κB induced by all the agents (Fig. 2A). Changes in the concentration of WS-2 and the time of exposure had minimal effect on cell viability. These results suggest that WS-2 acts at a step in the NF-κB activation pathway that is common to all four agents.

Inhibition of NF-κB Activation by Withanolide Is Dose Dependent

Because TNF is one of the most potent activators of NF-κB and the mechanism of activation of NF-κB is relatively well established (22), we examined the effect of withanolide on TNF-induced NF-κB activation in human myeloid KBM-5 cells. WS-2 suppressed TNF-induced NF-κB activation in a dose-dependent manner, with complete inhibition occurring at 5 μmol/L WS-2 (Fig. 2B).

Inhibition of NF-κB Activation by Withanolide Is Not Cell Type Specific

Because the signal transduction pathway mediated by NF-κB may be distinct in different cell types (23, 24), we also investigated whether withanolide could block TNF-induced NF-κB activation in breast adenocarcinoma MCF-7 cells (Fig. 2C). Withanolide completely inhibited most of the TNF-induced NF-κB activation, thus indicating that WS-2-induced suppression of NF-κB activation was not cell type specific.

Withanolide Also Suppresses Constitutive NF-κB Activation

Most tumor cells express constitutively active NF-κB (20, 21), although the mechanism of constitutive activation is not well understood. Human multiple myeloma (U266) cells, for example, are known to express constitutively active NF-κB (25, 26). We showed that WS-2 suppresses constitutive activation of NF-κB in U266 cells (Fig. 2D).

Suppression of NF-κB by Withanolide Is Time Dependent

To determine the minimum time of exposure to WS-2 required to inhibit TNF-mediated NF-κB activation, cells were exposed to the inhibitor 30, 15, or 5 minutes before TNF treatment, at the same time as TNF treatment, or 5, 15, or 30 minutes after TNF treatment. Figure 2E shows that maximum inhibition occurred when cells were exposed to WS-2 30 minutes before TNF exposure.

Withanolides Does Not Directly Interfere with the Binding of NF-κB to the DNA

Several NF-κB inhibitors suppress NF-κB activation by directly modifying the NF-κB protein, such that it can no longer bind to the DNA (27–29). When we incubated nuclear extracts from TNF-treated cells with WS-2, EMSA showed that WS-2 had no direct effect on NF-κB binding to the DNA (Fig. 2F). Thus, WS-2 must inhibit NF-κB activation through an indirect mechanism.

Withanolide Is a Potent Inhibitor of NF-κB Activation

To determine the effect of withanolide on NF-κB activation by higher concentrations of TNF, cells were treated with the indicated concentrations of TNF for 30 minutes in the absence or presence of WS-2 and then analyzed for NF-κB activation using EMSA (Fig. 2G). WS-2 abolished TNF-induced NF-κB activation even when TNF at a concentration of 10 nmol/L was used. These results show that WS-2 is a very potent inhibitor of TNF-induced NF-κB activation.

Withanolide Inhibits TNF-Dependent IκBα Degradation

We examined WS-2 for its effects on IκBα by Western blot analysis. WS-2 suppressed TNF-induced IκBα degradation (Fig. 3B) in synchrony with its suppression of TNF-induced NF-κB activation WS-2 (Fig. 3A).

Withanolide Inhibits TNF-Dependent IκBα Phosphorylation

Because IκBα phosphorylation is needed for IκBα degradation, we determined whether WS-2 modulated IκBα phosphorylation. Because TNF-induced phosphorylation of IκBα leads to its rapid degradation, we blocked IκBα degradation with the proteasome inhibitor *N*-acetyl-Leu-Leu-norleucinal. Western blot analysis using an antibody that is specific for the serine-phosphorylated form of IκBα showed that WS-2 suppressed TNF-induced phosphorylation of IκBα (Fig. 3C).

Withanolide Inhibits TNF-Induced IKK Activation

IKK is required for TNF-induced phosphorylation of IκBα (9) and for the phosphorylation of p65 (30). Because WS-2 inhibited the phosphorylation of IκBα, we determined its effect on TNF-induced IKK activation. Immune complex kinase assays show that WS-2 suppressed the activation of IKK by TNF (Fig. 3D). Neither TNF nor WS-2 had any effect on the expression of IKK proteins. To evaluate whether WS-2 suppresses IKK activity directly by binding to the IKK protein or by suppressing the activation of IKK, we incubated whole-cell extracts from untreated and TNF-treated cells with various concentrations of WS-2. Immune complex kinase assay showed that WS-2 did not directly affect the activity of IKK, suggesting that WS-2 modulates TNF-induced IKK activation (Fig. 3E).

Withanolide Inhibits TNF-Induced Nuclear Translocation of p65

As shown in Fig. 3F, Western blot analysis indicated that WS-2 significantly inhibited TNF-induced nuclear translocation of p65. Immunocytochemistry seemed to confirm this finding (Fig. 3G).

Withanolide Inhibits TNF-Induced Phosphorylation of p65

TNF also induces the phosphorylation of p65, which is required for its transcriptional activity (31). As shown in Fig. 3H, the coincubation of cells with WS-2 consistently inhibited TNF-induced phosphorylation of p65.

Withanolide Suppresses TNF-Induced NF-κB-Dependent Reporter Gene Expression

Because DNA binding does not always correlate with NF-κB-dependent gene transcription (32), we investigated the effect of WS-2 on TNF-induced reporter activity. Cells transiently transfected with the NF-κB-regulated SEAP reporter construct, incubated with WS-2, and then stimulated with TNF had significantly diminished reporter gene expression compared with cells that were not incubated withWS-2 (Fig. 4A). These results suggest that WS-2 inhibited TNF-induced gene expression.

Withanolide Inhibits NF-κB Activation Induced by TNFR1, TRADD, TRAF2, NIK, and IKK

TNF-induced NF-κB activation is mediated through sequential interaction of the TNFR with TRADD, TRAF2, NIK, and IKK, resulting in phosphorylation of IκBα (33, 34). When we transiently transfected cells with the NF-κB-regulated SEAP reporter construct, along with TNFR1-, TRADD-, TRAF2-, NIK-, IKK-β-, or p65-expressing plasmids, treated them with WS-2, and then monitored NF-κB-dependent SEAP expression, we found that WS-2 suppressed NF-κB activation induced by TNFR1, TRADD, TRAF2, NIK, and IKK-β but not that induced by p65 (Fig. 4A). These results suggested that WS-2 acts upstream of p65.

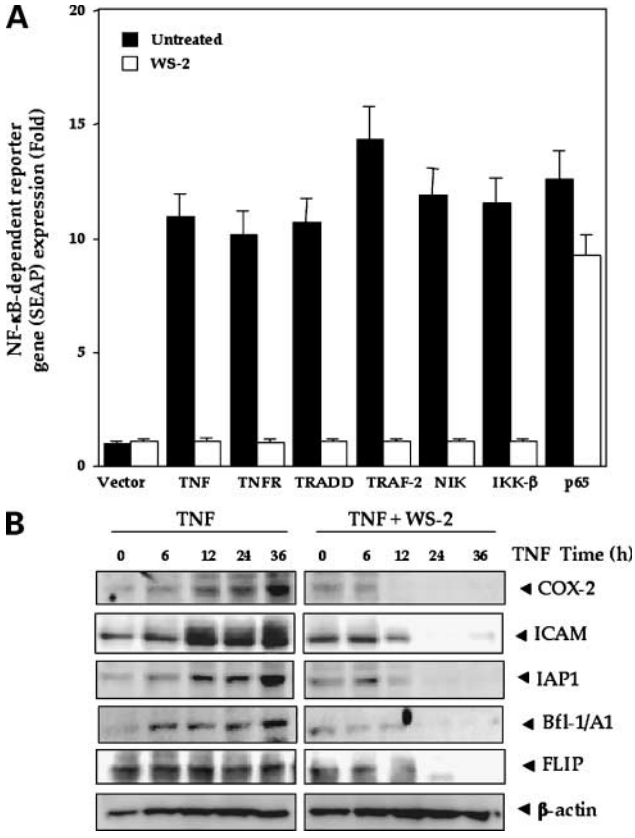

Figure 4. Withanolide inhibits the TNF-induced expression of the NF-κB-dependent genes TNFR1, TRADD, TRAF2, NIK, and IKK-β but not p65. **A,** A293 cells were transiently transfected with a NF-κB-containing SEAP reporter gene plasmid alone or with indicated plasmids for 24 h. After transfection, cells were washed and treated with 5 μmol/L WS-2 for 30 min. For TNF-treated cells, cells were treated with 1 nmol/L TNF for an additional 24 h. The supernatants of the culture medium were assayed for SEAP activity as described in Materials and Methods. **B,** withanolide inhibits the TNF-induced expression of NF-κB-dependent antiproliferation, antimetastatic, and antiapoptotic proteins. Withanolide inhibits expression of TNF-induced COX-2, intercellular adhesion molecule (*ICAM*), inhibitor of apoptosis protein 1 (*IAP1*), Bfl-1/A1, and FADD-like IL-1β-converting enzyme–inhibitory protein (*FLIP*). KBM-5 cells were preincubated with 1 μmol/L WS-2 for 30 min and 1 nmol/L TNF for the indicated times. Whole-cell extracts were prepared and analyzed by Western blot analysis using the indicated antibodies.

Withanolide Inhibits TNF-Induced NF-κB-Regulated Gene Products

WS-2 abolished TNF-induced expression of COX-2 (Fig. 4B), which is a NF-κB-regulated gene product (35, 36). NF-κB up-regulates the expression of several

genes implicated in facilitating tumor cell survival, including c-inhibitor of apoptosis protein 1 (37, 38), Bfl-1/A1 (39, 40), and c-FADD-like IL-1β-converting enzyme–inhibitory protein (41). We found that WS-2 inhibited the TNF-induced expression of all of these proteins (Fig. 4B).

Withanolide Potentiates Apoptosis Induced by TNF and Chemotherapeutic Agents

The activation of NF-κB can inhibit TNF-induced apoptosis (42–46), so we determined the potential of withanolide to enhance apoptosis induced by TNF and other cytotoxic agents using the live and dead assay, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide, poly(ADP-ribose) polymerase cleavage, and terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining methods. We first established that WS-2 enhanced the cytotoxicity induced by TNF (Fig. 5A1) and Taxol (Fig. 5A2). WS-2 by itself had little cytotoxic effect. Next, we showed that WS-2 enhanced cytotoxicity by potentiating TNF-induced apoptosis.

As shown in Fig. 5B, WS-2 potentiated the TNF-induced activation of caspases as indicated by the results of the poly(ADP-ribose) polymerase cleavage assay. The Live and Dead assay results indicated that WS-2 up-regulated TNF-induced cytotoxicity from 2% to 22% (Fig. 5C). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling staining showed that TNF-induced apoptosis was enhanced by incubation with WS-2 (Fig. 5D). In this assay, WS-2 alone exhibited cytotoxicity. The results of all the assays taken together suggest that WS-2 enhanced cytotoxicity by enhancing the apoptotic effects of TNF and Taxol.

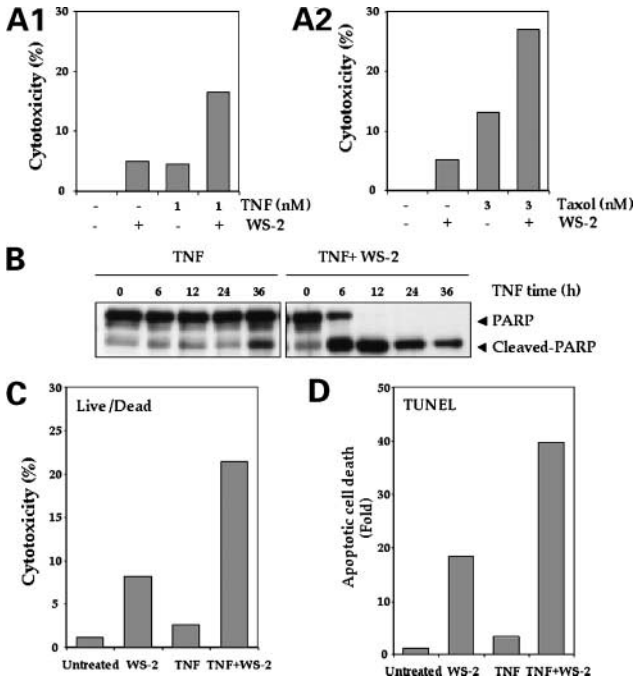

Figure 5. Withanolide enhances cytotoxicity. **A1,** withanolide enhances TNF-induced cytotoxicity. Ten thousand KBM-5 cells were seeded in triplicate in 96-well plates. Cells were coincubated with 1 μmol/L WS-2 and with indicated concentrations of TNF for 24 h. **A2,** withanolide enhances Taxol-induced cytotoxicity. KBM-5 cells were pretreated with 0.1 μmol/L WS-2 and then incubated with indicated concentrations of Taxol for 24 h. Thereafter, cell viability was analyzed by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide method as described in Materials and Methods. **B,** withanolide potentiates TNF-induced apoptosis. KBM-5 cells were preincubated with 1 μmol/L WS-2 for 30 min and then treated with 1 nmol/L TNF for the indicated times. Whole-cell extracts were prepared, subjected to SDS-PAGE, and blotted with anti–poly(ADP-ribose) polymerase (*PARP*) antibody. **C,** Live and Dead assay results indicate that withanolide up-regulates TNF-induced cytotoxicity. KBM-5 cells were preincubated with 1 μmol/L WS-2 for 30 min and then treated with 1 nmol/L TNF for 16 h. Cells were stained with live and dead assay reagent for 30 min and then analyzed under a fluorescence microscope as described in Materials and Methods. **D,** terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (*TUNEL*) staining shows that TNF-induced apoptosis is enhanced by incubation with WS-2. KBM-5 cells were preincubated with 1 μmol/L WS-2 for 30 min and then treated with 1 nmol/L TNF for 16 h. Cells were fixed, stained with terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay reagent, and then analyzed by flow cytometry as described in Materials and Methods.

Withanolide Suppresses TNF-Induced Invasion Activity

Matrix metalloproteinases, COXs, and adhesion molecules—all regulated by NF-κB—have been shown to mediate tumor invasion (47), and TNF can induce expression of genes involved in tumor metastasis (48). Whether withanolide modulates TNF-induced invasion activity *in vitro* was examined. For this experiment, we used H1299 cells seeded in the top chamber of a Matrigel invasion chamber in the absence of serum. Cells were incubated with TNF in the presence or absence of WS-2 for 24 hours. As shown in Fig. 6A, WS-2 suppressed TNF induced cell invasion activity.

Withanolide Inhibits RANKL-Induced Osteoclastogenesis in RAW 264.7 Cells

Recently, RANKL, a member of the TNF superfamily, has been implicated as a major mediator of bone resorption (49). Thus, agents that can suppress RANKL signaling have the potential to inhibit bone resorption or osteoclastogenesis. To determine the effect of withanolide on osteoclastogenesis, we incubated RAW 264.7 cells with 0.5 μmol/L WS-2 in the presence of RANKL and allowed them to grow and differentiate into osteoclasts. Figure 6B illustrates that WS-2 significantly decreased RANKL-induced differentiation of osteoclasts. A 0.5 μmol/L concentration of WS-2 was sufficient to reduce osteoclastogenesis by >30%. Under these conditions, the cells remained fully viable (data not shown).

Discussion

The present study was designed to determine the effects of withanolide on the NF-κB activation pathway and on NF-κB-regulated gene products that control inflammation,

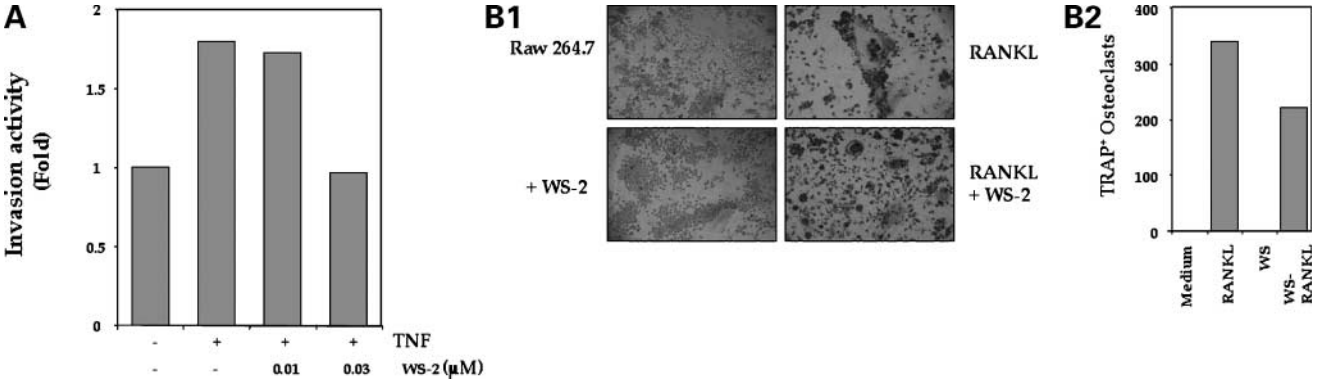

Figure 6. **A,** withanolide suppresses TNF-induced invasion activity. H1299 cells were seeded to the top chamber of a Matrigel invasion chamber overnight in the absence of serum, preincubated with indicated WS-2 for 30 min, treated with 1 nmol/L TNF for 24 h in the presence of 1% serum, and then subjected to invasion assay as described in Materials and Methods. **B,** withanolide inhibits RANKL-induced osteoclastogenesis. RAW 264.7 cells were incubated either alone or in the presence of RANKL (5 nmol/L) with or without 0.5 μmol/L WS-2 for 5 d and stained for TRAP expression. **B1,** TRAP-positive cells were photographed. Original magnification, ×100. **B2,** multinucleated (three nuclei) osteoclasts were counted.

tumor cell survival, invasion, and osteoclastogenesis. We found that withanolide suppressed NF-κB activation induced by a wide variety of inflammatory and carcinogenic agents. Both inducible and constitutive NF-κB activation was blocked. Inhibition of NF-κB by withanolide occurred through inhibition of IKK activation, IκBα phosphorylation, IκBα degradation, p65 phosphorylation, p65 nuclear translocation, and NF-κB-dependent reporter gene expression activation. Suppression of NF-κB-regulated antiapoptotic gene products enhanced apoptosis and suppressed cellular TNF-induced invasion and RANKL-induced osteoclastogenesis (Fig. 7).

This is the first report to examine the effect of withanolide on NF-κB activated by various stimuli. Our results indicate that withanolide suppresses NF-κB activated by carcinogens, tumor promoters, and inflammatory stimuli in a variety of cell lines, suggesting that withanolide must act at a molecular step common to all these agents. We found that withanolide blocked the activation of NF-κB without directly interfering with the DNA binding of NF-κB. This inhibition was mediated through inhibition of IKK by withanolide, which led to the suppression of phosphorylation and degradation of IκBα. Withanolide also inhibited the TNF-induced phosphorylation of p65, nuclear p65 translocation, and NF-κB-dependent reporter gene activity. Our *in vitro* kinase assay results show that withanolide is not a direct inhibitor of IKK. Thus, it seems that this agent blocks the activation of IKK by interfering with some upstream regulatory kinases. Akt, NIK, mitogen-activated protein kinase 1, and atypical protein kinase C are all candidates because they are upstream kinases that regulate IKK (8). It is possible that one of these kinases is modulated by withanolides.

We found that withanolide inhibited not only inducible NF-κB activation but also constitutively activated NF-κB in multiple myeloma cells. Constitutive NF-κB activation has been found to be critical for the survival and proliferation of various tumor cell types (8); however, the mechanism of constitutive NF-κB activation is not well understood. Some of the potential mechanisms are

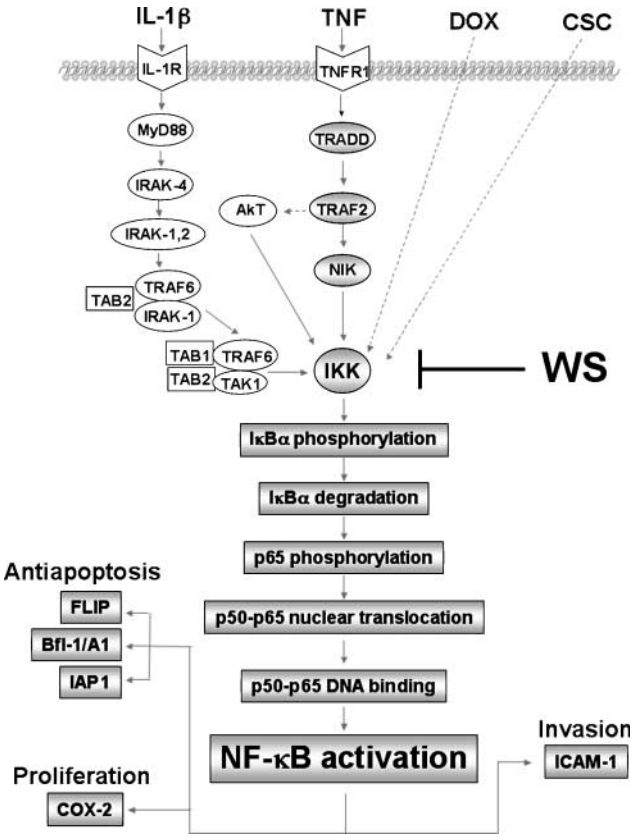

Figure 7. Proposed mechanism by which withanolide inhibits NF-κB activation and NF-κB-regulated gene expression involved cell proliferation and invasion.

overexpression of IκBα without inhibition of NF-κB activity, mutations in the *IκBα* gene, enhanced IκBα degradation, and constitutive expression of TNF and IL-1 (8).

Genes involved in the proliferation and metastasis of cancer have been shown to be regulated by NF-κB (8). Our results show that the expression of COX-2, which is regulated by NF-κB, is down-regulated by withanolide. Activation of NF-κB is known to promote angiogenesis and invasion, and withanolide is antiangiogenic (4) and antimetastatic (5); it is possible that the antiangiogenic effects of withanolide are mediated through suppression of NF-κB. In agreement with these observations, we also found that TNF-induced invasion is inhibited by withanolide. It is also possible that the chemopreventive (50–55) and radiosensitizing (56–60) effects of withanolide described previously are also mediated through the inhibition of NF-κB.

NF-κB is known to regulate the expression of inhibitor of apoptosis protein 1, Bfl-1/A1, and c-FADD-like IL-1β-converting enzyme–inhibitory protein, and their overexpression in numerous tumors has been linked to tumor survival, chemoresistance, and radioresistance. Our results indicate that withanolide down-regulates most of these gene products. Earlier studies have shown that withanolide inhibits proliferation of various tumor cells (19) and induces apoptosis. It is possible that down-regulation of NF-κB-regulated genes, as described here, is linked to the growth-modulatory effects of this agent. Our results also show that withanolide potentiated the apoptotic effects of TNF and Taxol. These effects are similar to those of a specific inhibitor of NF-κB (15). We also found that withanolide inhibited TNF-induced invasion and RANKL-induced osteoclastogenesis.

That withanolide can suppress adjuvant-induced arthritis has been reported (61). Because NF-κB activation can mediate arthritis (62–64), it is possible that this effect occurs through the suppression of NF-κB activation. There are numerous reports indicating that withanolides exhibit cardioprotective effects (65–67). Because NF-κB activation has been linked with cardiovascular diseases (68, 69), it is possible that the cardioprotective effects of withanolide are mediated through the suppression of NF-κB as well. Whether all the effects of withanolides reported here and described previously are due to down-regulation of NF-κB-regulated gene expression is not clear. We have shown previously that withanolides can directly suppress COX-2 activity (2), indicating targets other than NF-κB. Future investigations may reveal more targets through which withanolides mediate their multiple effects.

Overall, our results suggest that the antiproliferative, proapoptotic, anti-invasive, antiosteoclastogenic, antiangiogenic, antimetastatic, radiosensitizing, antiarthritic, and cardioprotective effects assigned to withanolide may be mediated in part through the suppression of NF-κB and NF-κB-regulated gene products.

Acknowledgments

We thank Dawn Chalaire for a careful review of the article and Dr. Bryant Darnay for supplying the RANKL protein.

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