

# Pinitol targets nuclear factor- $\kappa$ B activation pathway leading to inhibition of gene products associated with proliferation, apoptosis, invasion, and angiogenesis

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## Abstract

Pinitol (3-*O*-methyl-chiroinositol), a component of traditional Ayurvedic medicine (talisapatra), has been shown to exhibit anti-inflammatory and antidiabetic activities through undefined mechanisms. Because the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B) has been linked with inflammatory diseases, including insulin resistance, we hypothesized that pinitol must mediate its effects through modulation of NF- $\kappa$ B activation pathway. We found that pinitol suppressed NF- $\kappa$ B activation induced by inflammatory stimuli and carcinogens. This suppression was not specific to cell type. Besides inducible, pinitol also abrogated constitutive NF- $\kappa$ B activation noted in most tumor cells. The suppression of NF- $\kappa$ B activation by pinitol occurred through inhibition of the activation of I $\kappa$ B $\alpha$  kinase, leading to sequential suppression of I $\kappa$ B $\alpha$  phosphorylation, I $\kappa$ B $\alpha$  degradation, p65 phosphorylation, p65 nuclear translocation, and NF- $\kappa$ B-dependent reporter gene expression. Pinitol also suppressed the NF- $\kappa$ B reporter activity induced by tumor necrosis factor receptor (TNFR)-1, TNFR-associated death domain, TNFR-associated factor-2, transforming growth factor- $\beta$ -activated kinase-1 (TAK-1)/TAK1-binding protein-1, and I $\kappa$ B $\alpha$  kinase but not that induced by p65. The inhibition of NF- $\kappa$ B activation thereby led to down-regulation of gene products involved in inflammation (cyclooxygenase-2), proliferation (cyclin D1 and c-myc), invasion (matrix metalloproteinase-9), angiogenesis (vascular endothelial growth factor), and cell survival (cIAP1, cIAP2, X-linked inhibitor apoptosis pro-

tein, Bcl-2, and Bcl-xL). Suppression of these gene products by pinitol enhanced the apoptosis induced by TNF and chemotherapeutic agents and suppressed TNF-induced cellular invasion. Our results show that pinitol inhibits the NF- $\kappa$ B activation pathway, which may explain its ability to suppress inflammatory cellular responses. [Mol Cancer Ther 2008;7(6):1604–14]

## Introduction

Traditional medicine, whether Chinese, Ayurvedic, Kampo, or Unani, is being used by a majority of the people of the world even today, when “rational drug design,” “targeted therapies,” and “smart drugs” are the buzz words in pharmacotherapy. The problems with drugs used in allopathic medicine are that, with some exceptions, they are highly toxic, very expensive, and most of the time not very effective (1–4). Although some of these therapies are monotargeted for a specific disease, most diseases exhibit dysregulation of multiple targets. In most cancers, for instance, as many as 300 to 500 gene products have been shown to be dysregulated (1). Traditional medicines, on the other hand, generally are safe and inexpensive, but often neither their active constituents nor their mechanisms of action are well defined (5).

One such traditional remedy, talisapatra (called morinda in Hindi), derived from the plant *Abies pindrow* (Pinacea), is described in Ayurveda for various respiratory and inflammatory ailments. The active component, pinitol (3-*O*-methyl-chiroinositol), has been identified from this and other plants (e.g., *Bougainvillea spectabilis*). Pinitol has been also linked with inhibition of the T-helper cell-1 response (6) and has antiviral (7) and larvicidal (8) activities. This alicyclic polyalcohol exerts insulin-like effects and has been shown to lower blood glucose concentrations in diabetic rats (9) and in human subjects (9–12). Pinitol also possesses anti-inflammatory properties (13, 14) and has been implicated in the prevention of cardiovascular diseases (11). In experimental animal models of inflammation, pinitol has been shown to be effective against carrageenan-induced paw edema (13, 14) and ovalbumin-induced airway inflammation in a murine model of asthma (15).

How pinitol mediates many of these effects is unclear. Because most genes that cause inflammation are regulated by the transcription factor nuclear factor- $\kappa$ B (NF- $\kappa$ B), we postulated that pinitol must mediate its effects through modulation of NF- $\kappa$ B activation. NF- $\kappa$ B is a family of Rel domain-containing proteins present in the cytoplasm of all cells, where they are kept in an inactive state by a family of anchorin domain-containing proteins that includes inhibitory subunit of NF- $\kappa$ B $\alpha$  (I $\kappa$ B $\alpha$ ), I $\kappa$ B $\beta$ , I $\kappa$ B $\gamma$ , I $\kappa$ B $\epsilon$ , bcl-3, p105, and p100. Under resting conditions, NF- $\kappa$ B consists of

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a heterotrimer of p50, p65, and I $\kappa$ B $\alpha$  in the cytoplasm (16); only when it is activated and translocated to the nucleus is the sequence of events leading to activation initiated. Most carcinogens, inflammatory agents, and tumor promoters, including cigarette smoke, phorbol ester, okadaic acid, and tumor necrosis factor (TNF), have been shown to activate NF- $\kappa$ B (17). The activation of NF- $\kappa$ B involves phosphorylation, ubiquitination, and degradation of I $\kappa$ B $\alpha$  and phosphorylation of p65, which in turn lead to translocation of NF- $\kappa$ B to the nucleus, where it binds to specific response elements in the DNA (18). The phosphorylation of I $\kappa$ B $\alpha$  is catalyzed by I $\kappa$ B $\alpha$  kinase (IKK), which is essential for NF- $\kappa$ B activation by most agents.

We investigated whether pinitol exerts its anti-inflammatory effects through suppression of the NF- $\kappa$ B pathway and found that pinitol inhibited activation of NF- $\kappa$ B through abrogation of IKK activation, I $\kappa$ B $\alpha$  phosphorylation and degradation, p65 nuclear translocation, DNA binding, and NF- $\kappa$ B-dependent reporter gene expression. The suppression of NF- $\kappa$ B by pinitol inhibited TNF-induced cell invasion and led to down-regulation of gene products that prevent apoptosis and promote inflammation and tumor metastasis.

## Materials and Methods

### Materials

Pinitol was obtained from Sigma-Aldrich. A 50 mmol/L solution of pinitol was prepared in 100% DMSO, stored as small aliquots at -20°C, and then diluted as needed in cell culture medium. Bacteria-derived recombinant human TNF, purified to homogeneity with a specific activity of  $5 \times 10^7$  units/mg, was kindly provided by Genentech. Cigarette smoke condensate, prepared as previously described, was kindly supplied by Dr. C.G. Gairola (University of Kentucky; ref. 19). Penicillin, streptomycin, Iscove's modified Dulbecco's medium, and fetal bovine serum were obtained from Invitrogen. Phorbol 12-myristate 13-acetate, okadaic acid, and anti- $\beta$ -actin antibody were obtained from Sigma-Aldrich. Antibodies against p65, p50, I $\kappa$ B $\alpha$ , cyclin D1, matrix metalloproteinase-9 (MMP-9), poly(ADP-ribose) polymerase (PARP), inhibitor of apoptosis proteins 1 (IAP1) and 2 (IAP2), Bcl-2, Bcl-xL, and glyceraldehyde-3-phosphate dehydrogenase were obtained from Santa Cruz Biotechnology. Antibodies against cyclooxygenase-2 (COX-2) and X-linked IAP were obtained from BD Biosciences. Phosphospecific anti-I $\kappa$ B $\alpha$  (Ser<sup>32</sup>/Ser<sup>36</sup>) and phosphospecific anti-p65 (Ser<sup>536</sup>) antibodies were purchased from Cell Signaling Technology. Anti-IKK $\alpha$  and anti-IKK $\beta$  antibodies were kindly provided by Imgenex.

### Cell Lines

Human myeloid KBM-5 cells, human lung adenocarcinoma H1299 cells, human multiple myeloma U266 cells, and human embryonic kidney A293 cells were obtained from the American Type Culture Collection. KBM-5 cells were cultured in Iscove's modified Dulbecco's medium supplemented with 15% fetal bovine serum. H1299 and U266 cells were cultured in RPMI 1640, and A293 cells were

cultured in DMEM supplemented with 10% fetal bovine serum. All media were supplemented with 100 units/mL penicillin and 100  $\mu$ g/mL streptomycin.

### Cytotoxicity Assay

Cytotoxicity was assayed by the modified tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described elsewhere (20).

### PARP Cleavage Assay

For detection of cleavage products of PARP, whole-cell extracts were prepared by subjecting pinitol-treated cells to lysis in lysis buffer [20 mmol/L Tris (pH 7.4), 250 mmol/L NaCl, 2 mmol/L EDTA (pH 8.0), 0.1% Triton X-100, 0.01  $\mu$ g/mL aprotinin, 0.005  $\mu$ g/mL leupeptin, 0.4 mmol/L phenylmethylsulfonyl fluoride, 4 mmol/L NaVO<sub>4</sub>]. Lysates were spun at 14,000 rpm for 10 min to remove insoluble material, subjected to 10% SDS-PAGE, and probed with PARP antibody.

### Live/Dead Assay

To measure apoptosis, we used the Molecular Probes Live/Dead assay (Invitrogen), which determines intracellular esterase activity and plasma membrane integrity. This assay uses calcein, a polyanionic dye, which is retained within live cells and yields green fluorescence. It also uses the ethidium monomer dye (red fluorescence), which can enter cells only through damaged membranes and bind to nucleic acids but is excluded by the intact plasma membrane of live cells. Briefly,  $1 \times 10^5$  cells were incubated with pinitol and then treated with TNF at 37°C. Cells were stained with the Live/Dead reagent (5  $\mu$ mol/L ethidium homodimer, 5  $\mu$ mol/L calcein-AM) and then incubated at 37°C for 30 min. Cells were analyzed under a fluorescence microscope (Labophot-2; Nikon).

### Invasion Assay

The membrane invasion culture system was used to assess cell invasion, because invasion through the extracellular matrix is a crucial step in tumor metastasis. The BD BioCoat Tumor Invasion system is a chamber that has a light-tight polyethylene terephthalate membrane with 8- $\mu$ m pores and is coated with a reconstituted basement membrane gel (BD Biosciences). A total of  $2.5 \times 10^4$  cells were suspended in serum-free medium and seeded into the upper wells. After incubation overnight, cells were treated with pinitol and then stimulated with TNF in the presence of 1% fetal bovine serum and pinitol. The cells that invaded through the Matrigel (those that migrated to the lower chamber during incubation) were stained with 4  $\mu$ g/mL calcein-AM (Invitrogen) in PBS for 30 min at 37°C and scanned for fluorescence with a Victor 3 multiplate reader (Perkin-Elmer Life and Analytical Sciences); fluorescent cells were counted.

### Annexin V Assay

One of the early indicators of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cell's cytoplasmic interface to the extracellular surface. This loss of membrane asymmetry can be detected using the binding properties of Annexin V. To identify apoptosis, we used an Annexin V

antibody, which was conjugated with the FITC fluorescence dye. Briefly,  $5 \times 10^5$  cells were pretreated with pinitol, treated with TNF for 16 h at 37°C, and subjected to Annexin V staining. The cells were washed in PBS, resuspended in 100  $\mu$ L binding buffer containing a FITC-conjugated anti-Annexin V antibody, and then analyzed with a flow cytometer (FACSCalibur; BD Biosciences).

#### Electrophoretic Mobility Shift Assay

To determine NF- $\kappa$ B activation by TNF, we did electrophoretic mobility shift assay (EMSA) essentially as described elsewhere (21). Briefly, nuclear extracts prepared from TNF-treated cells ( $1 \times 10^6$ /mL) were incubated with [ $^{32}$ P]end-labeled 45-mer double-stranded 5  $\mu$ mol/L oligonucleotide (15  $\mu$ g protein with 16 fmol DNA) from the HIV long terminal repeat, 5'-TTGTTACAAGG-GACTTTCGCTGGGGACTTTCAGGGAGGCGTGG-3' (italics indicates 5  $\mu$ mol/L binding sites), for 30 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'-TTGTTACA ACTCACTTTCGCTGCTCACTTTCAGG-GGGAGGCGTGG-3', was used to examine the specificity of binding of NF- $\kappa$ B to the DNA. The dried gels were visualized with a Storm820 PhosphorImager, and radioactive bands were quantified by using ImageQuant software (Amersham Biosciences).

#### Western Blot Analysis

To determine the effect of pinitol on TNF-dependent I $\kappa$ B $\alpha$  phosphorylation and degradation, cytoplasmic extracts were prepared as described elsewhere (20) from cells that had been pretreated with pinitol and then exposed to TNF for various times. Cytoplasmic protein (30  $\mu$ g) was resolved by 10% SDS-PAGE, transferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with specific antibodies against I $\kappa$ B $\alpha$  and phosphorylated I $\kappa$ B $\alpha$ . To determine the expression of various NF- $\kappa$ B-regulated gene products in whole-cell extracts of TNF- and pinitol-treated cells, 50  $\mu$ g protein was resolved by SDS-PAGE and probed by Western blot with specific antibodies per the manufacturer's recommended protocol. The blots were washed, exposed to horseradish peroxidase-conjugated secondary antibodies for 1 h, and finally detected by the enhanced chemiluminescence reagent (Amersham Pharmacia Biotechnology). The bands were quantified by using a Personal Densitometer Scan v1.30 and ImageQuant software v3.3.

#### IKK Assay

To determine the effect of pinitol on TNF-induced IKK activation, we analyzed IKK by a method described elsewhere (22). Briefly, the IKK complex from whole-cell extracts was precipitated with antibodies against IKK $\alpha$  and IKK $\beta$  then treated with protein A/G-agarose beads (Pierce). After 2 h, the beads were washed with lysis buffer and then resuspended in a kinase assay mixture containing 50 mmol/L HEPES (pH 7.4), 20 mmol/L MgCl $_2$ , 2 mmol/L DTT, 20  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP, 10  $\mu$ mol/L unlabeled ATP, and 2  $\mu$ g substrate GST-I $\kappa$ B $\alpha$  (amino acids 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 subjected to 10% SDS-PAGE, the gel was dried, and the radioactive bands were visualized with a Storm820. To determine the total amounts of IKK $\alpha$  and IKK $\beta$  in each sample, 50  $\mu$ g whole-cell protein was subjected to 7.5% SDS-PAGE, electrotransferred to a nitrocellulose membrane, and then blotted with either anti-IKK $\alpha$  or anti-IKK $\beta$  antibody.

#### NF- $\kappa$ B-Dependent Reporter Gene Transcription

The effect of pinitol on TNF-induced NF- $\kappa$ B-dependent reporter gene transcription in A293 cells was measured as described elsewhere (22).

## Results

The goal of this study was to investigate whether pinitol affects NF- $\kappa$ B activation, NF- $\kappa$ B-regulated gene products, or NF- $\kappa$ B-mediated cellular responses. The structure of pinitol (Fig. 1A) indicates that it is a methyl chiroinositol. The concentration of pinitol used and the duration of exposure had minimal effect on the viability of cells as determined by the trypan blue dye exclusion test. For most studies, human myeloid KBM-5 cells were used because these cells have been shown to express both types of TNF receptors (TNFR). To examine the effects of pinitol on the NF- $\kappa$ B activation pathway, most studies used TNF because the pathway activated by this agent is relatively well understood.

#### Pinitol Blocks NF- $\kappa$ B Activation Induced by Various Anti-inflammatory and Carcinogenic Agents

We investigated whether pinitol modulates NF- $\kappa$ B activation induced by various agents. Cells pretreated with pinitol were exposed to various NF- $\kappa$ B activators, including TNF, phorbol 12-myristate 13-acetate, okadaic acid, lipopolysaccharide, and cigarette smoke condensate. DNA-binding assay (EMSA) showed that pinitol suppressed the NF- $\kappa$ B activation induced by all these agents (Fig. 1B). These results suggest that pinitol acts at a step in the NF- $\kappa$ B activation pathway that is common to all these agents.

#### Pinitol Suppresses NF- $\kappa$ B Activation in a Dose- and Time-Dependent Manner

We then determined the dose and time of exposure to pinitol required to suppress NF- $\kappa$ B activation. The EMSA results showed that pinitol alone had no effect on NF- $\kappa$ B activation. However, it inhibited TNF-mediated NF- $\kappa$ B activation in a dose-dependent manner (Fig. 1C). The suppression of NF- $\kappa$ B activation by pinitol was also found to be time dependent (Fig. 1D).

#### Inhibition of NF- $\kappa$ B Activation by Pinitol Is Not Cell Type Specific

It has been reported that the NF- $\kappa$ B induction pathway in epithelial cells may differ from that in lymphoid cells (23). We therefore investigated whether pinitol inhibited NF- $\kappa$ B activation in different cell types. Pinitol completely inhibited TNF-induced NF- $\kappa$ B activation in lung adenocarcinoma (H1299) cells and embryonic kidney (A293) cells (Fig. 2A), indicating a lack of cell type specificity.

### Pinitol Inhibits Constitutive NF- $\kappa$ B Activation

Why some tumor cells express constitutive active NF- $\kappa$ B and others do not is not fully characterized. Whether pinitol affects constitutive NF- $\kappa$ B activation in human multiple myeloma (U266) tumor cells was examined (24). Pinitol inhibited constitutively active NF- $\kappa$ B in a dose-dependent manner (Fig. 2B). These results indicated that pinitol is a broad-acting inhibitor of NF- $\kappa$ B.

### Pinitol Does Not Directly Affect Binding of NF- $\kappa$ B to DNA

Some NF- $\kappa$ B inhibitors directly modify NF- $\kappa$ B to suppress its DNA binding (25–28). We examined whether pinitol mediates its effect through such a mechanism. EMSA showed that pinitol did not modify the DNA-binding ability of NF- $\kappa$ B proteins prepared from TNF-treated cells (Fig. 2C). These results suggest that pinitol inhibits NF- $\kappa$ B activation by a mechanism different than that of some other agents.

### Pinitol Inhibits TNF-Dependent I $\kappa$ B $\alpha$ Degradation

Because I $\kappa$ B $\alpha$  degradation is required for activation of NF- $\kappa$ B (29), we investigated whether inhibition of TNF-induced NF- $\kappa$ B activation by pinitol was due to inhibition of I $\kappa$ B $\alpha$  degradation. We found that TNF induced I $\kappa$ B $\alpha$  degradation in control cells as early as 10 min after treatment, but in pinitol-pretreated cells TNF had no effect on I $\kappa$ B $\alpha$  degradation (Fig. 3A).

### Pinitol Inhibits TNF-Dependent I $\kappa$ B $\alpha$ Phosphorylation

We then determined whether pinitol affected the TNF-induced I $\kappa$ B $\alpha$  phosphorylation needed for I $\kappa$ B $\alpha$  degradation. We used *N*-acetyl-leucyl-leucyl-norleucinal, which prevents degradation of phosphorylated I $\kappa$ B $\alpha$ . Western blot analysis using an antibody that detects only the Ser<sup>32</sup>/Ser<sup>36</sup> phosphorylated form of I $\kappa$ B $\alpha$  indicated that TNF induced I $\kappa$ B $\alpha$  phosphorylation and that pinitol completely suppressed it (Fig. 3B). Thus, pinitol inhibited TNF-induced NF- $\kappa$ B activation by inhibiting phosphorylation and degradation of I $\kappa$ B $\alpha$ .

### Pinitol Inhibits TNF-Induced IKK Activation

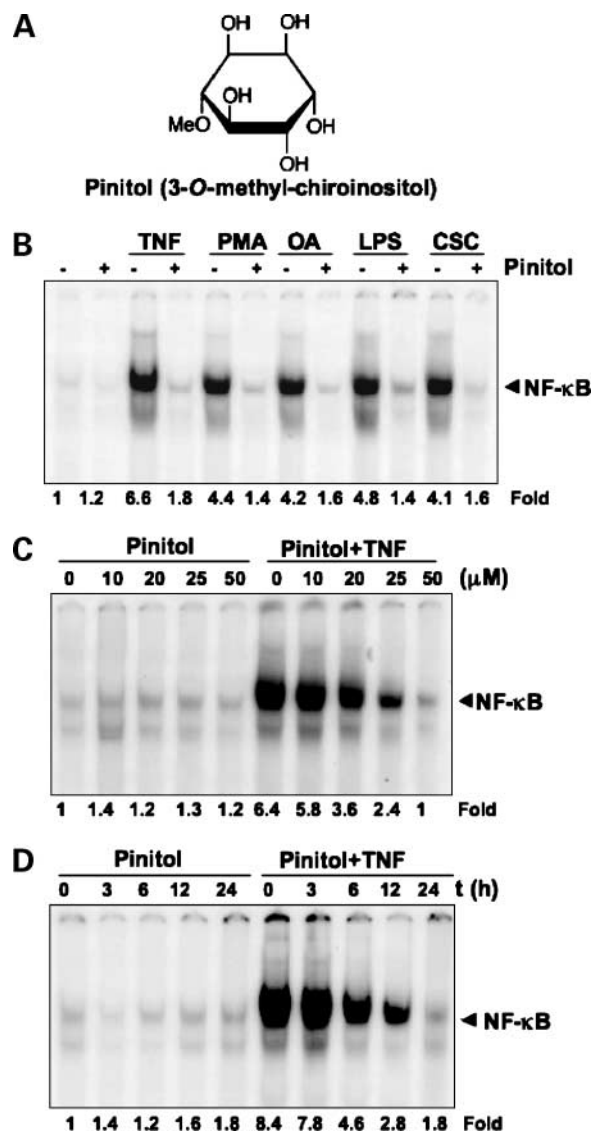
Because pinitol inhibits phosphorylation of I $\kappa$ B $\alpha$ , we tested its effect on TNF-induced IKK activation, which is required for TNF-induced phosphorylation of I $\kappa$ B $\alpha$ . As shown in Fig. 3C (left), pinitol completely suppressed TNF-induced activation of IKK. Neither TNF nor pinitol had any direct effect on the expression of IKK $\alpha$  and IKK $\beta$  proteins. An immunocomplex kinase assay showed that pinitol had no direct effect on the activity of IKK, suggesting that pinitol modulates TNF-induced IKK activation (Fig. 3C, right).

### Pinitol Inhibits TNF-Induced Nuclear Phosphorylation and Translocation of p65

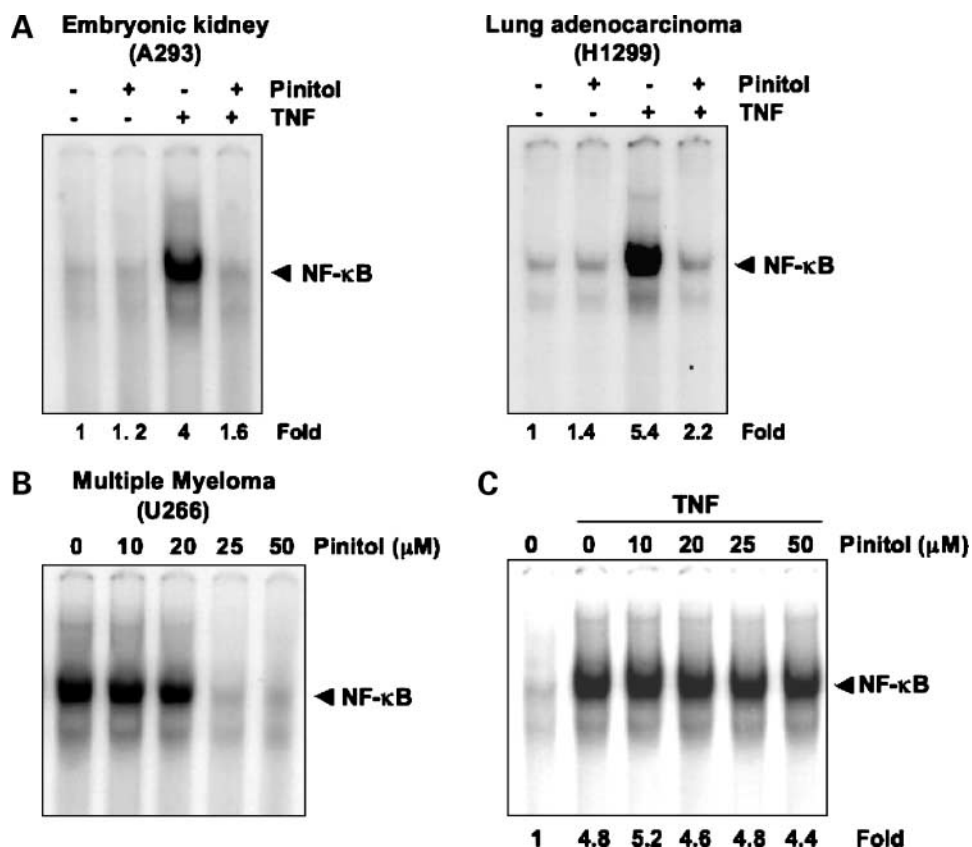
We also tested the effect of pinitol on TNF-induced phosphorylation of p65, because phosphorylation is required for transcriptional activity of p65 (30). As shown in Fig. 3D (top), pinitol suppressed p65 phosphorylation almost completely. We also examined by Western blot analysis the effect of pinitol on TNF-induced nuclear translocation of p65. As shown in Fig. 3D, pinitol inhibited nuclear translocation of the p65 subunit of NF- $\kappa$ B.

### Pinitol Represses TNF-Induced NF- $\kappa$ B-Dependent Reporter Gene Expression

We examined whether pinitol modulates NF- $\kappa$ B-dependent gene transcription by transiently transfecting the



**Figure 1.** **A**, chemical structure of pinitol. **B**, pinitol blocks NF- $\kappa$ B activation induced by TNF, phorbol 12-myristate 13-acetate, okadaic acid, lipopolysaccharide, and cigarette smoke condensate. Human myeloid leukemia KBM-5 cells were preincubated with 50  $\mu$ M pinitol for 12 h and then treated with 0.1 nmol/L TNF for 30 min, 25 ng/mL phorbol 12-myristate 13-acetate for 1 h, 500 nmol/L okadaic acid for 4 h, 10  $\mu$ g/mL lipopolysaccharide for 1 h, or 10  $\mu$ g/mL cigarette smoke condensate for 1 h. Nuclear extracts were analyzed for NF- $\kappa$ B activation as described in Materials and Methods. Representative of three independent experiments. **C**, effect of pinitol dose. KBM-5 cells were incubated with the indicated concentrations of pinitol for 12 h and treated with 0.1 nmol/L TNF for 30 min. Nuclear extracts were assayed for NF- $\kappa$ B activation by EMSA. Representative of three independent experiments. **D**, effect of duration of pinitol exposure. KBM-5 cells were preincubated with 50  $\mu$ M pinitol for the indicated times and then treated with 0.1 nmol/L TNF for 30 min. Nuclear extracts were prepared and assayed for NF- $\kappa$ B activation by EMSA. Representative of three independent experiments.



**Figure 2.** **A**, pinitol suppresses TNF-induced NF- $\kappa$ B activation in different cell types. Human lung adenocarcinoma H1299 (*left*) and human embryonic kidney A293 (*right*) cells were incubated with 50  $\mu$ mol/L pinitol for 12 h followed by incubation with 0.1 nmol/L TNF for 30 min. Nuclear extracts were then prepared and assayed for NF- $\kappa$ B activation by EMSA. Representative of three independent experiments. **B**, pinitol suppresses constitutive NF- $\kappa$ B activation in multiple myeloma cells. U266 cells were incubated with indicated concentrations of pinitol for 12 h; nuclear extracts were then prepared and assayed for NF- $\kappa$ B activation by EMSA. Representative of three independent experiments. **C**, direct effect of pinitol on the NF- $\kappa$ B complex was investigated. Nuclear extracts were prepared from untreated cells or cells treated with 0.1 nmol/L TNF and incubated for 30 min with the indicated concentrations of pinitol. They were then assayed for NF- $\kappa$ B activation by EMSA. Representative of three independent experiments.

cells with the NF- $\kappa$ B. We found that TNF produced an ~5-fold increase in NF- $\kappa$ B-dependent expression of secretory alkaline phosphatase (SEAP) activity over vector control (Fig. 4A), which was inhibited by dominant-negative I $\kappa$ B $\alpha$ , indicating specificity. When the cells were pretreated with pinitol, TNF-induced NF- $\kappa$ B-dependent expression of SEAP was inhibited in a dose-dependent manner (Fig. 4A). These results showed that pinitol inhibits the NF- $\kappa$ B-dependent reporter gene expression induced by TNF.

We next determined where pinitol acts in the sequence of TNFR1, TNFR-associated death domain, TNFR-associated factor 2, NF- $\kappa$ B-inducing kinase (5), transforming growth factor- $\beta$ -activated kinase 1 (TAK1)/TAB1-binding protein (TAB1), and IKK recruitment that characterizes TNF-induced NF- $\kappa$ B activation (31). In cells transfected with TNFR1, TNFR-associated death domain, TNFR-associated factor 2, TAK1/TAB1, IKK $\beta$ , or p65 plasmids, NF- $\kappa$ B-dependent reporter gene expression was induced; pinitol suppressed SEAP expression in all cells, except those transfected with p65 (Fig. 4B).

#### **Pinitol Suppresses TNF-Induced NF- $\kappa$ B-Dependent Gene Products Involved in Cell Proliferation**

Numerous gene products that mediate cellular proliferation, such as cyclin D1, COX-2, and c-myc, have NF- $\kappa$ B-binding sites in their promoters (32–34). We investigated whether expression of these gene products is modulated by pinitol. Western blot analysis indicated that TNF induced

the expression of these proteins and pinitol suppressed it (Fig. 5A). These results suggest that pinitol may suppress cellular proliferation through suppression of NF- $\kappa$ B-regulated gene products.

#### **Pinitol Represses Expression of TNF-Induced NF- $\kappa$ B-Dependent Antiapoptotic Gene Products**

NF- $\kappa$ B regulates expression of the antiapoptotic proteins IAP1/2 (35, 36), X-linked IAP (37), Bcl-2 (38), and Bcl-xL (39). We investigated whether pinitol could modulate TNF-induced expression of these antiapoptotic gene products. The results of Western blot analysis showed that TNF induced expression of these antiapoptotic proteins in a time-dependent manner and that pinitol suppressed it (Fig. 5B).

#### **Pinitol Suppresses TNF-Induced NF- $\kappa$ B-Dependent Gene Products Involved in Invasion and Angiogenesis**

Gene products that have been linked with invasion (MMP-9) and angiogenesis [vascular endothelial growth factor (VEGF)] are known to be regulated by NF- $\kappa$ B (40, 41). We examined whether expression of these gene products is modulated by pinitol. Western blot analysis indicated that TNF induced these gene products and that pinitol suppressed this expression (Fig. 5C). These results suggest a role for pinitol in blocking invasion and angiogenesis.

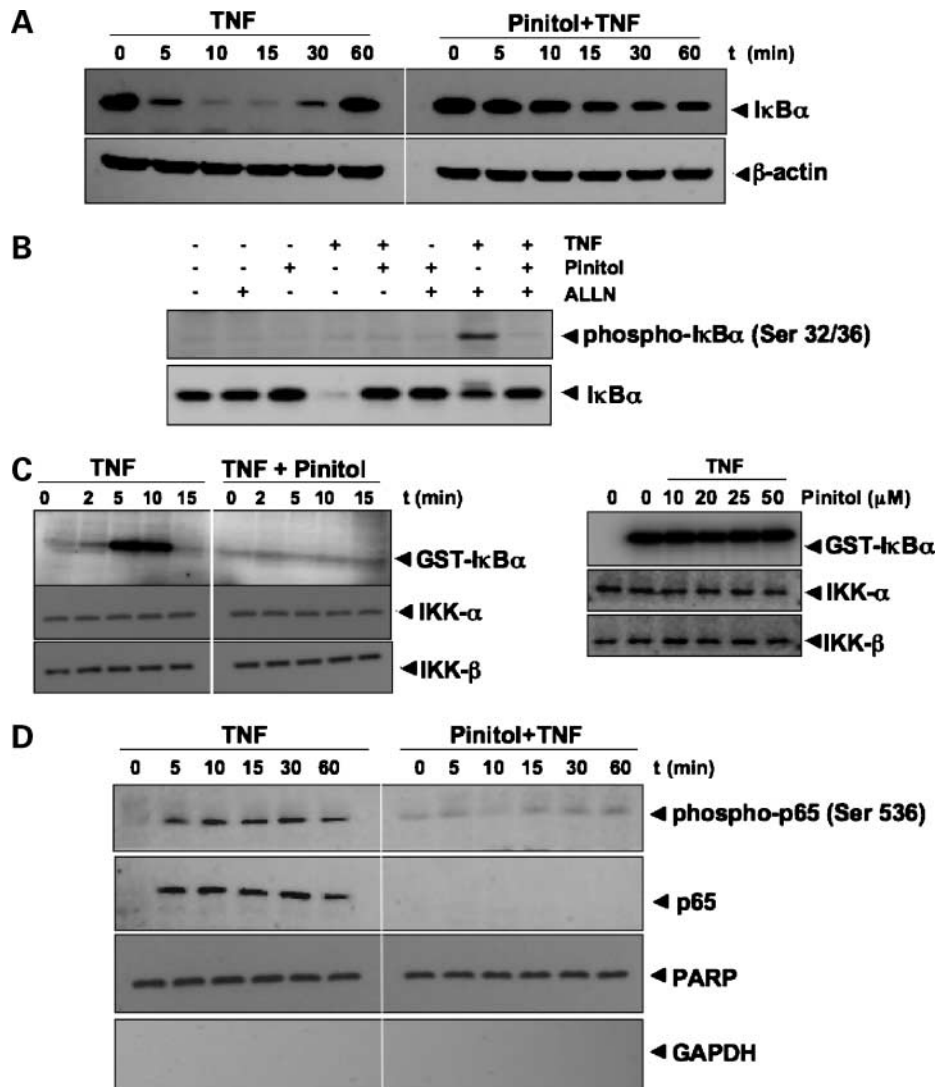
#### **Pinitol Alone Had Minimal Effect on Cell Proliferation in Different Cell Types**

Because pinitol suppressed TNF-induced activation of NF- $\kappa$ B and NF- $\kappa$ B-regulated gene products, whether it

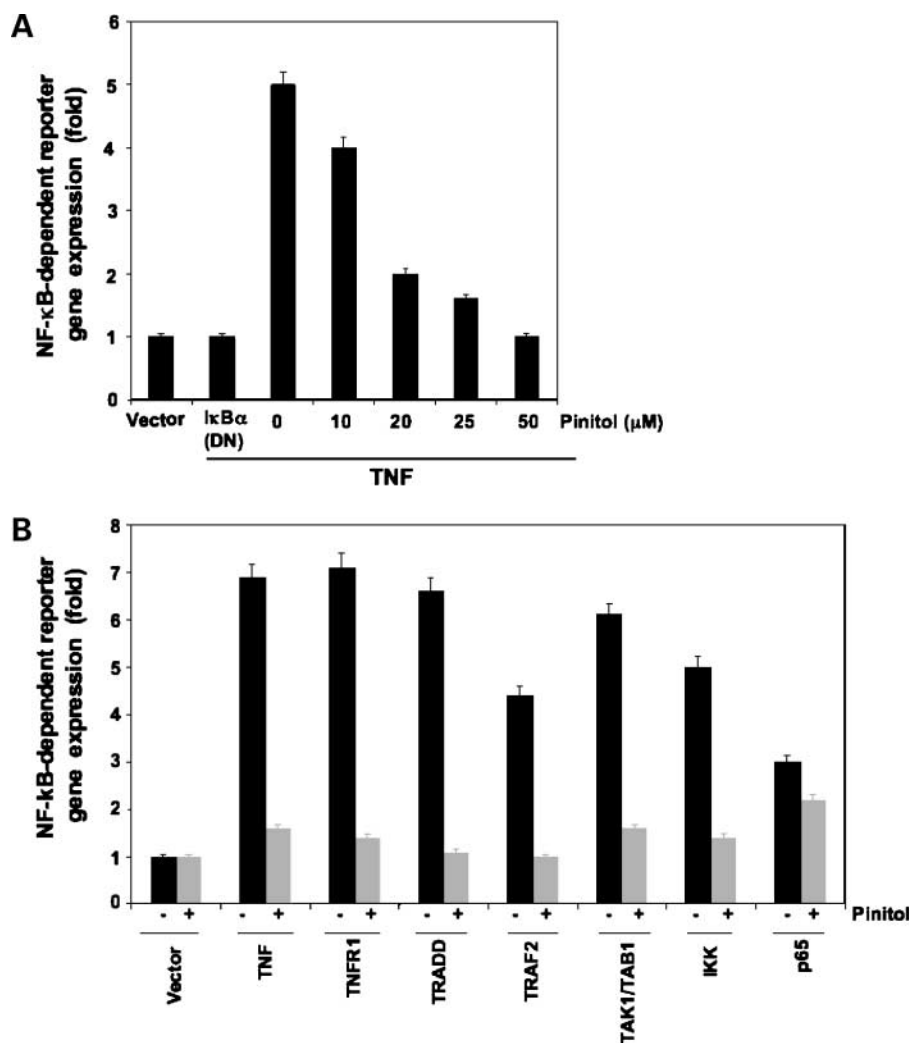
alone modulates proliferation of various cell types was examined. Results in Fig. 5D show that pinitol alone had minimal effect on the proliferation of KBM-5, U266, and H1299 cells. Perhaps maximum suppression was observed on day 3 with KBM-5 cells.

### Pinitol Potentiates the Apoptotic Effects of TNF and Chemotherapeutic Drugs

Because NF- $\kappa$ B activation has been shown to suppress apoptosis induced by various agents (42, 43), we investigated whether pinitol would modulate the apoptosis



**Figure 3.** **A**, effect of pinitol on TNF-induced degradation of IκBα. KBM-5 cells were incubated with 50 μmol/L pinitol for 12 h and treated with 0.1 nmol/L TNF for the indicated times. Cytoplasmic extracts (CE) were prepared and analyzed by Western blotting using antibodies against IκBα. Representative of three independent experiments. β-Actin was used to check for equal protein loading. **B**, effect of pinitol on the phosphorylation by IκBα by TNF. Cells were preincubated with 50 μmol/L pinitol for 12 h, incubated with 50 μg/mL *N*-acetyl-leucyl-leucyl-norleucinal for 30 min, and then treated with 0.1 nmol/L TNF for 10 min. Cytoplasmic extracts were fractionated and then subjected to Western blot analysis using phosphospecific IκBα antibody. The same membrane was reblotted with β-actin antibody. **C**, effect of pinitol on activation of IKK by TNF. KBM-5 cells were preincubated with 50 μmol/L pinitol for 12 h, incubated with 50 μg/mL *N*-acetyl-leucyl-leucyl-norleucinal for 30 min, and then treated with 1 nmol/L TNF for the indicated times. Whole-cell extracts were immunoprecipitated with antibodies against IKKα and IKKβ and analyzed by an immune complex kinase assay using GST-IκBα as a substrate. To examine the effect of pinitol on the level of expression of IKK proteins, whole-cell extracts were fractionated on SDS-PAGE and examined by Western blot analysis using anti-IKKα and anti-IKKβ antibodies. Representative of three independent experiments (*left*). Direct effect of pinitol on IKK activation induced by TNF. Whole-cell extracts were prepared from KBM-5 cells treated with 1 nmol/L TNF and immunoprecipitated with anti-IKKα and IKK-β antibodies. The immunocomplex kinase assay was done in the absence or presence of the indicated concentration of pinitol (*right*). **D**, pinitol inhibits TNF-induced phosphorylation and nuclear translocation of p65. KBM-5 cells were either untreated or pretreated with 50 μmol/L pinitol for 12 h at 37°C and then treated with 0.1 nmol/L TNF for the indicated times. Nuclear extracts (NE) were prepared and analyzed by Western blotting using antibodies against phosphospecific p65 and p65. Representative of three independent experiments. To examine the purity of the nuclear extracts, gels were stripped and reprobbed with antibodies against glyceraldehyde-3-phosphate dehydrogenase (cytoplasmic marker) and against PARP (nuclear marker).



**Figure 4.** **A**, pinitol inhibits TNF-induced NF- $\kappa$ B-dependent reporter gene (SEAP) expression. A293 cells were transiently transfected with a NF- $\kappa$ B-containing plasmid linked to the SEAP gene and then treated with the indicated concentrations of pinitol. After 24 h in culture with 1 nmol/L 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**, pinitol inhibits NF- $\kappa$ B-dependent reporter gene expression induced by TNFR, TNFR-associated death domain, TNFR-associated factor, NIK, IKK $\beta$ , p65, and TAK1/TAK1-binding protein. A293 cells were transiently transfected with the indicated plasmids along with a NF- $\kappa$ B-containing plasmid linked to the SEAP gene and then were either left untreated or treated with 50  $\mu$ mol/L pinitol 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, SD.

induced by TNF and by chemotherapeutic agents. We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay to examine the effect of pinitol on apoptosis induced by TNF and chemotherapeutic agents and found that pinitol enhanced the cytotoxic effects of TNF, paclitaxel, and doxorubicin in all three different cell types (Fig. 6A).

The Live/Dead assay, which measures intracellular esterase activity and plasma membrane integrity, indicated that pinitol up-regulated TNF-induced apoptosis from 8% to 72% (Fig. 6B, left). Similarly, Annexin V staining showed that pinitol is quite effective in enhancing the effects of TNF (Fig. 6B, right). By using caspase-3-activated PARP cleavage, we showed that the enhanced cytotoxicity was due to apoptosis. TNF-induced PARP cleavage was enhanced in pinitol-treated cells by caspase-3 activation (Fig. 6C).

#### Pinitol Suppresses TNF-Induced Tumor Cell Invasion Activity

Because pinitol suppresses the expression of MMP-9, which mediates tumor cell invasion, we investigated whether it can modulate tumor cell invasion activity

*in vitro*. Human lung adenocarcinoma H1299 cells, which have been known to exhibit high invasive activity, were seeded into the top chamber of the Matrigel invasion chamber with TNF in the presence or absence of pinitol and their invasiveness was examined. The result showed that TNF induced tumor cell invasion by  $\sim$ 4-fold, but pinitol suppressed this activity (Fig. 6D, left). Because down-regulation of MMP-9 by pinitol earlier was examined in KBM-5 cells, whether expression of MMP-9 is modulated by pinitol in the H1299 tumor cells was examined. Western blot analysis indicated that TNF induced the gene product and that pinitol suppressed this expression (Fig. 6D, right). These results suggest a down-regulation of MMP-9 in H1299 cells by pinitol is linked to the inhibition of invasion.

#### Discussion

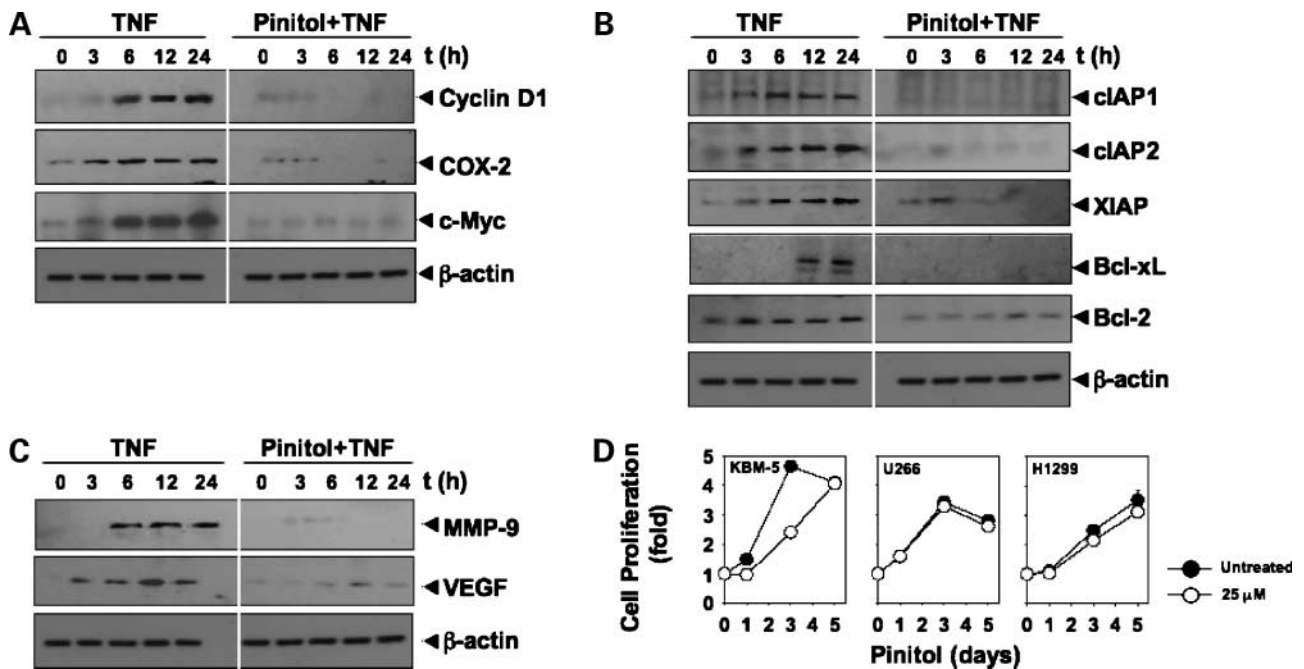
Various anti-inflammatory activities traditionally ascribed to pinitol suggest that it must mediate its effects by suppressing NF- $\kappa$ B activation pathway. In the study presented here, we found that this inositol indeed inhibited

activation of NF- $\kappa$ B induced by numerous agents and in a variety of cell lines. We found that pinitol suppressed IKK activation, resulting in inhibition of I $\kappa$ B $\alpha$  phosphorylation and degradation. Consequently, this polyalcohol blocked p65 phosphorylation, nuclear translocation, and gene transcription. Expression of gene products involved in cell proliferation, antiapoptosis, and invasion, were all inhibited, thus enhancing apoptosis and reducing cellular invasion.

This is the first report to examine the effects of pinitol on NF- $\kappa$ B activated by various stimuli. Our results indicate that activation of NF- $\kappa$ B by a wide variety of inflammatory agents and carcinogens was abrogated. These findings suggest that pinitol acts at a step common to all these agents. Pinitol did not block NF- $\kappa$ B activation by direct modification of the p65 subunit of NF- $\kappa$ B, as most agents do (25–28), but by suppressing nuclear translocation of p65. Our results agree with a report that showed the ability of pinitol to inhibit lipopolysaccharide-induced nuclear p65 in dendritic cells (15). Various tumor cells express a constitutively activated form of NF- $\kappa$ B through a mechanism that is not fully understood (16). This carbohydrate also suppressed constitutive NF- $\kappa$ B activation in our study.

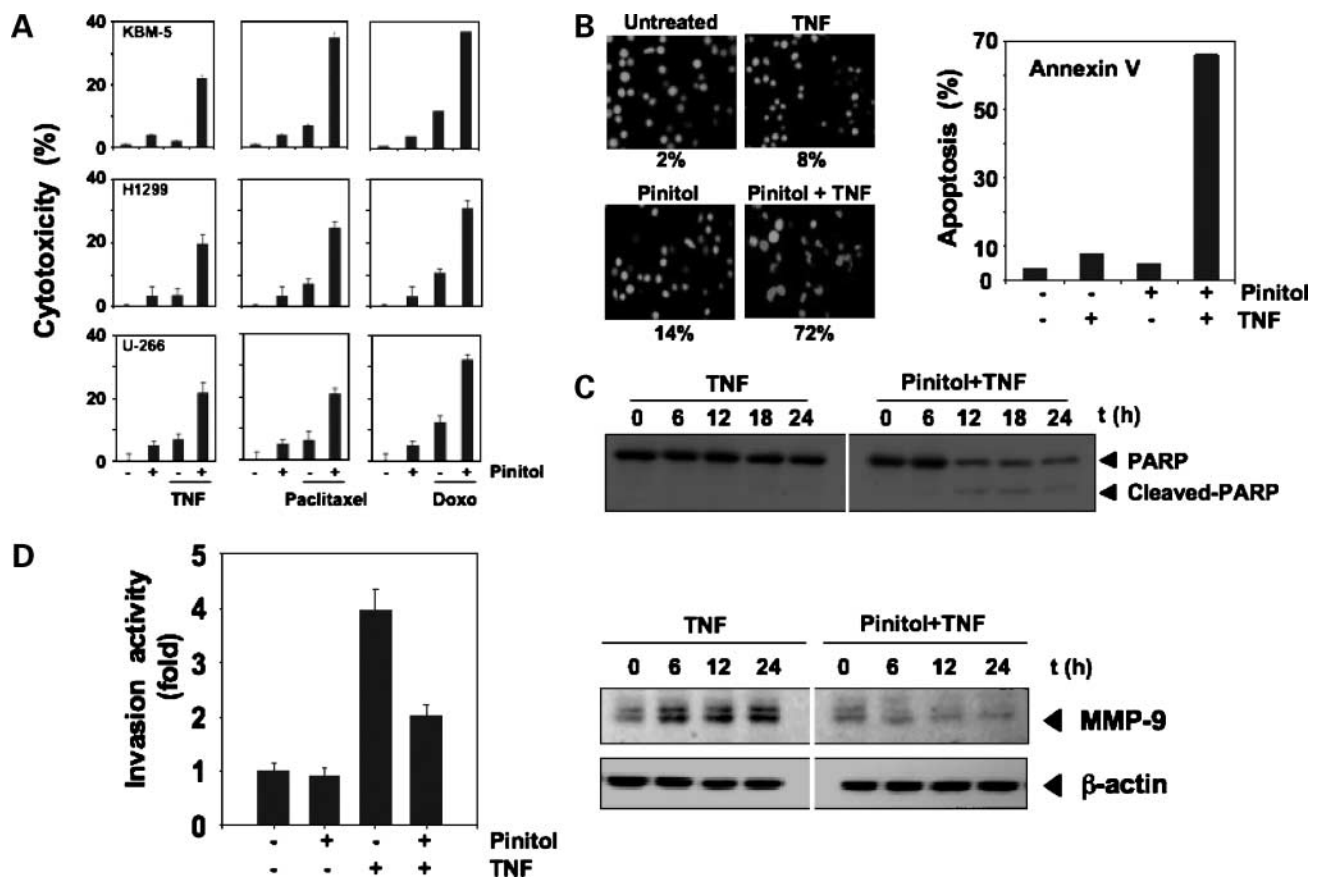
Because pinitol also inhibited TNF-induced phosphorylation and degradation of I $\kappa$ B $\alpha$ , we reasoned that it may mediate its inhibitory effect through IKK, the kinase required for I $\kappa$ B $\alpha$  phosphorylation. Indeed, we found that pinitol suppressed TNF-induced IKK activation and did not directly inhibit IKK activity. It is possible that this inhibition is the result of inhibition of an upstream kinase. Recent studies have shown that TAK1 is a major kinase directly responsible for activation of IKK pathway (44, 45). We also found that pinitol suppresses NF- $\kappa$ B-dependent reporter gene expression. It suppressed NF- $\kappa$ B activation induced by overexpression of TNFR1, TNFR-associated death domain, TNFR-associated factor 2, and IKK plasmids but had no effect on activation induced by p65 plasmid. These results suggest that pinitol acts at a step between IKK and p65. Indeed, pinitol suppressed TAK1/TAK1-binding protein-induced NF- $\kappa$ B-dependent reporter gene expression. These results suggest that pinitol-induced inhibition of IKK activation may be mediated through the modulation of upstream kinases such as TAK1. Our data also suggest that pinitol had no effect on the expression of IKK protein.

In our study, pinitol down-regulated the expression of NF- $\kappa$ B-regulated gene products involved in cell proliferation



**Figure 5.** Pinitol inhibits TNF-induced NF- $\kappa$ B-regulated gene products. **A**, pinitol suppresses cyclin D1, COX-2, and c-Myc expression induced by TNF. KBM-5 cells were left untreated or were incubated with 25  $\mu$ mol/L pinitol for 12 h and then treated with 1 nmol/L TNF for different times. Whole-cell extracts were prepared, and 30  $\mu$ g whole-cell lysate was analyzed by Western blot analysis using antibodies against cyclin D1, COX-2, or c-Myc. **B**, pinitol inhibits the expression of antiapoptotic gene products. KBM-5 cells were left untreated or were incubated with 25  $\mu$ mol/L pinitol for 12 h and then treated with 1 nmol/L TNF for different amounts of time. Whole-cell extracts were prepared, and 30  $\mu$ g whole-cell lysate was analyzed by Western blotting using antibodies against IAP1, IAP2, X-linked IAP, Bcl-xL, and Bcl-2 as indicated. **C**, pinitol inhibits MMP-9 and VEGF expression induced by TNF. KBM-5 cells were left untreated or were incubated with 25  $\mu$ mol/L pinitol for 12 h and then treated with 1 nmol/L TNF for different times. Whole-cell extracts were prepared, and 30  $\mu$ g whole-cell lysate was analyzed by Western blot analysis using antibodies against MMP-9 or VEGF. Representative of three independent experiments showing similar results. **D**, pinitol suppresses cell proliferation in different type cancer cells. Cells (2,000 per well) were plated in triplicate, treated with 25  $\mu$ mol/L pinitol, and then subjected to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay on days 0 to 5 to analyze proliferation of cells. Representative of three independent experiments.





**Figure 6.** **A**, pinitol enhances TNF-induced and chemotherapeutic agent – induced cytotoxicity. In total, 10,000 cells were seeded in triplicate in 96-well plates. KBM-5, H1299, and U266 cells were pretreated with 25  $\mu$ mol/L pinitol for 12 h and then incubated with the indicated concentrations of TNF, paclitaxel, or doxorubicin for 24 h. Cell viability was analyzed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method as described in Materials and Methods. **B**, pinitol potentiates TNF-induced apoptosis. KBM-5 cells were pretreated with 25  $\mu$ mol/L pinitol for 12 h and then incubated with 1 nmol/L TNF for 24 h. The cells were stained with a Live/Dead assay reagent for 30 min and then analyzed under a fluorescence microscope as described in Materials and Methods. Representative of three independent experiments (*left*). KBM-5 cells were pretreated with 25  $\mu$ mol/L pinitol for 12 h and then incubated with 1 nmol/L TNF for 16 h. The cells were incubated with a FITC-conjugated Annexin V antibody and then analyzed by flow cytometry as described in Materials and Methods. Representative of two independent experiments (*left*). **C**, KBM-5 cells were pretreated with 25  $\mu$ mol/L pinitol for 12 h and then incubated with 1 nmol/L TNF for the indicated times. Whole-cell extracts were prepared and analyzed by Western blotting using an anti-PARP antibody. Representative of three independent experiments. **D**, pinitol suppresses TNF-induced invasion activity. H1299 cells ( $2.5 \times 10^4$ ) were seeded into the top chamber of a Matrigel invasion chamber system overnight in the absence of serum and then treated with 25  $\mu$ mol/L pinitol for 12 h. After incubation, the cells were treated with 1 nmol/L TNF for 16 h in the presence of 1% serum and then assayed for invasion as described in Materials and Methods. Results are expressed as fold activity of the untreated control (*left*). Pinitol inhibits MMP-9 expression induced by TNF. H1299 cells were left untreated or were incubated with 25  $\mu$ mol/L pinitol for 12 h and then treated with 1 nmol/L TNF for different times. Whole-cell extracts were prepared, and 30  $\mu$ g whole-cell lysate was analyzed by Western blot analysis using antibodies against MMP-9 (*right*).

(COX-2, cyclin D1, and c-Myc), invasion (MMP-9 and VEGF), and antiapoptosis (cIAP1, cIAP2, X-linked IAP, Bcl-2, and Bcl-xL). Whether suppression of proliferation genes (c-myc, cyclin D1, and COX-2) is due to inhibition of NF- $\kappa$ B activation or to suppression of other transcription factors such as  $\beta$ -catenin is not clear. Our group, however, has shown that deletion of NF- $\kappa$ B (p65) abolishes the TNF-induced expression of COX-2 and cyclin D1 (46), indicating the critical requirement of NF- $\kappa$ B in the expression of these genes. The suppression of expression of various NF- $\kappa$ B regulated gene products examined here may explain the antitumor and antiviral properties of recently synthesized analogues of pinitol (7, 47). We also found that this inositol potentiated the apoptotic effects of TNF and

chemotherapeutic agents. It is very likely that this potentiation is mediated through the suppression of antiapoptotic gene products regulated by NF- $\kappa$ B. Pinitol also suppressed TNF-induced tumor invasion. Invasion and metastasis require the expression of MMP-9 and COX-2, all of which were modulated by pinitol. VEGF expression was also suppressed by this inositol. Inhibitors of VEGF such as Avastin have been approved for the treatment of different cancers (2). The activity of pinitol against type 2 diabetes has been extensively documented. Because IKK- $\beta$  has been closely linked with type 2 diabetes (48–50), it is possible that inhibition of IKK as described here plays a major role in its antidiabetic activity. Similarly, its activity against carrageenan-induced paw edema (13, 14), and ovalbumin-induced

airway inflammation in a murine model of asthma (15), could also be due to its ability to suppress NF- $\kappa$ B pathway. Thus, these results suggest that pinitol is effective not only as an anti-inflammatory agent but also as a therapeutic agent through regulation of cell proliferation, apoptosis, invasion, and angiogenesis.

Besides tumorigenesis, NF- $\kappa$ B activation has been linked with numerous biological functions, thus raising the question about the safety of NF- $\kappa$ B inhibitors such as pinitol. Both *in vitro* and *in vivo* studies, however, suggest that pinitol is quite safe. For instance, *in vitro*, pinitol had no cytotoxic effects on normal bone marrow dendritic cells even at 80  $\mu$ mol/L dose (6). In animal studies, pinitol was found to be quite safe when given at 100 mg/kg (p.o. or i.p.) twice daily for 11 days (9) or 20 mg/kg/day i.p. for 20 days (15). In human subjects with type 2 diabetes mellitus, when administered at 1.2 g/d to examine the postprandial blood glucose response, pinitol was found to be well tolerated (51). Thus, these reports suggest that pinitol is quite safe compound.

Overall, our results suggest that pinitol is an effective blocker of the NF- $\kappa$ B pathway and thus may have potential in treatment of a wide variety of NF- $\kappa$ B-linked proinflammatory diseases (52). However, further studies are needed in animals to validate these findings for the therapeutic use of this agent.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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