Honokiol Potentiates Apoptosis, Suppresses Osteoclastogenesis, and Inhibits Invasion through Modulation of Nuclear Factor-κB Activation Pathway

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Abstract

Recent reports have indicated that honokiol can induce apoptosis, suppress tumor growth, and inhibit angiogenesis. In this report, we found that honokiol potentiated the apoptosis induced by tumor necrosis factor (TNF) and chemotherapeutic agents, suppressed TNF-induced tumor cell invasion, and inhibited RANKL-induced osteoclastogenesis, all of which are known to require nuclear factor-κB (NF-κB) activation. Honokiol suppressed NF-κB activation induced by a variety of inflammatory stimuli, and this suppression was not cell type specific. Further studies showed that honokiol blocked TNF-induced phosphorylation, ubiquitination, and degradation of IκBα through the inhibition of activation of IκBα kinase and of Akt. This led to suppression of the phosphorylation and nuclear translocation of p65 and NF-κB-dependent reporter gene expression. Magnolol, a honokiol isomer, was equally active. The expression of NF-κB-regulated gene products involved in antiapoptosis (IAP1, IAP2, Bcl-x_L, Bcl-2, cFLIP, TRAF1, and survivin), proliferation (cyclin D1, cyclooxygenase-2, and c-myc), invasion (matrix metalloproteinase-9 and intercellular adhesion molecule-1), and angiogenesis (vascular endothelial growth factor) were also down-regulated by honokiol. Honokiol also down-regulated NF-κB activation in *in vivo* mouse dorsal skin model. Thus, overall, our results indicate that NF-κB and NF-κB-regulated gene expression inhibited by honokiol enhances apoptosis and suppresses osteoclastogenesis and invasion. (Mol Cancer Res 2006;4(9):621–33)

Introduction

More than 80% of the world population cannot afford modern medicine and thus must rely on traditional medicine. Although practiced for centuries, traditional medicine suffers from the fact that both the chemical entity responsible for the activity and its molecular targets are not well defined. Nonetheless, as much as 70% of all drugs approved by the Food and Drug Administration for cancer between 1980 and 2000 have been from natural sources (1). Thus, identification of the chemical entity of traditional medicine and its targets can make such drugs most acceptable.

Honokiol, used for almost three decades as muscle relaxant (2), is derived from the stem and bark of the plant *Magnolia officinalis*, which is used in traditional Chinese and Japanese medicine. Extensive research has shown that honokiol inhibits skin tumor promotion (3), inhibits nitric oxide synthesis and tumor necrosis factor (TNF) expression (4, 5), inhibits invasion (6), down-regulates antiapoptotic protein Bcl-x_L (7), inhibits angiogenesis and tumor growth *in vivo* (8), induces caspase-dependent apoptosis in B-cell chronic lymphocytic leukemia cells through down-regulation of the antiapoptotic protein Mcl-1 (9), and overcomes drug resistance in multiple myeloma (10). Exactly how honokiol mediates all these effects is poorly understood.

Apoptosis, invasion, angiogenesis, and inflammation have been shown to be regulated by the nuclear transcription factor nuclear factor-κB (NF-κB; refs. 11, 12). NF-κB 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 ankyrin domain-containing proteins that includes IκBα, IκBβ, IκBγ, IκBε, Bcl-3, p105, and p100. Under resting conditions, NF-κB consists of a heterotrimer of p50, p65, and IκBα in the cytoplasm (13); however, when activated, this transcription factor translocates to the nucleus. Most carcinogens, inflammatory agents, and tumor promoters, including cigarette smoke, phorbol ester, okadaic acid, H_2O_2, and TNF, have been shown to activate NF-κB (14). The activation of NF-κB involves the phosphorylation, ubiquitination, and

Received 3/20/06; revised 6/23/06; accepted 6/27/06.
Grant support: Clayton Foundation for Research (B.B. Aggarwal), Department of Defense U.S. Army Breast Cancer Research Program grant BC010610 (B.B. Aggarwal), NIH on Lung Chemoprevention grant PO1 CA91844 (B.B. Aggarwal), and NIH Head and Neck Specialized Programs of Research Excellence grant P50 (B.B. Aggarwal).
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doi:10.1158/1541-7786.MCR-06-0076

degradation of IκBα and phosphorylation of p65, which in turn lead to the translocation of NF-κB to the nucleus where it binds to specific response elements in the DNA (15). The phosphorylation of IκBα is catalyzed by IκBα kinase (IKK), which is essential for NF-κB activation by most agents. NF-κB has been shown to regulate the expression of several genes whose products are involved in tumorigenesis (11). These include antiapoptotic genes (e.g., *cIAP, survivin, TRAF, cFLIP, Bfl-1, Bcl-2*, and *Bcl-x_L*); *cyclooxygenase-2* (*COX-2*); *matrix metalloproteinase-9* (*MMP-9*); *vascular endothelial growth factor* (*VEGF*); genes encoding adhesion molecules, chemokines, and inflammatory cytokines; and cell cycle regulatory genes (e.g., *cyclin D1* and *c-myc*).

Because honokiol regulates the expression of various gene products that are regulated by NF-κB and suppresses tumor cell proliferation, invasion, and angiogenesis, which are also regulated by NF-κB, we postulated that this agent must mediate its effects through modulation of the NF-κB activation pathway. To test this hypothesis, we investigated the effect of honokiol on NF-κB activation induced by a variety of inflammatory agents and carcinogens in various cell types. We found that honokiol inhibited the activation of NF-κB through inhibition of IκBα kinase, IκBα phosphorylation and degradation, p65 nuclear translocation, DNA binding, and NF-κB-dependent reporter gene expression. The suppression of NF-κB by honokiol inhibited TNF-induced cell invasion and abrogated RANKL-induced osteoclastogenesis. Honokiol also led to the down-regulation of gene products that prevent apoptosis and promote inflammation and tumor metastasis.

Results

The goal of this study was to investigate the effect of honokiol on the transcription factor NF-κB signaling pathway, on NF-κB-regulated gene products, and on NF-κB-mediated cellular responses. The structure of honokiol is shown in Fig. 1A. The concentration of honokiol used and the duration of exposure had minimal effect on the viability of cells as determined by the trypan blue dye exclusion test. To examine the effect of honokiol on the NF-κB activation pathway, TNF was used for most experiments, because the pathway activated by this agent is relatively well understood.

Honokiol Potentiates the Apoptotic Effects of TNF and Chemotherapeutic Drugs

Because NF-κB activation has been shown to suppress the apoptosis induced by various agents (16, 17), we investigated whether honokiol would modulate the apoptosis induced by TNF-induced and chemotherapeutic agents in KBM-5 cells. The effect of honokiol on TNF-induced and chemotherapeutic agent–induced apoptosis was examined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. We found that honokiol enhanced the cytotoxic effects of TNF, 5-fluorouracil paclitaxel, and doxorubicin (Fig. 1B).

By using caspase-activated poly(ADP-ribose) polymerase (PARP) cleavage, we showed that the enhanced cytotoxicity was due to apoptosis. TNF-induced PARP cleavage was enhanced in the honokiol-treated cells (Fig. 1C). The Live/Dead assay, which measures intracellular esterase activity and plasma membrane integrity, also indicated that honokiol up-regulated TNF-induced apoptosis from 5% to 65% (Fig. 1D). Similarly, Annexin V staining also showed that honokiol is quite effective in enhancing the effects of TNF (Fig. 1E). The results of all these assays together suggest that honokiol enhances the apoptotic effects of TNF and chemotherapeutic agents.

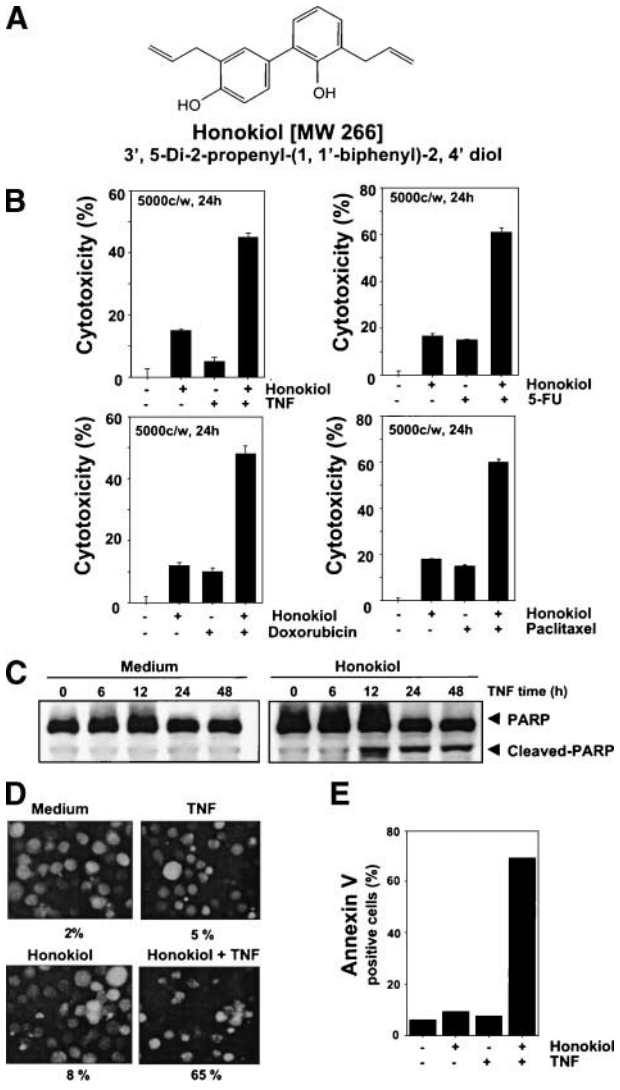


FIGURE 1. **A.** Structure of honokiol. **B.** Honokiol enhances apoptosis induced by TNF and chemotherapeutic agents. KBM-5 cells (5,000/0.1 mL) were incubated at 37°C with TNF, 5-FU paclitaxel, or doxorubicin in the presence and absence of 5 μmol/L honokiol for 72 hours, and the viable cells were assayed using the MTT reagent. Columns, mean cytotoxicity of triplicate cultures; bars, SD. **C.** Honokiol enhances TNF-induced PARP cleavage. KBM-5 cells (2×10^6/mL) were then incubated with TNF (1 nmol/L) alone or in combination with honokiol (25 μmol/L) for the indicated times, and PARP cleavage was determined by Western blot analysis as described in Materials and Methods. Densitometric analysis of the 87-kDa band. **D.** Honokiol enhances TNF-induced cell death. KBM-5 cells (2×10^6/mL) were incubated with TNF (1 nmol/L) alone or in combination with honokiol (10 μmol/L) for 24 hours. Cell death was determined by the calcein-AM-based Live/Dead assay as described in Materials and Methods. Representative of three independent experiments showing similar results. **E.** Honokiol up-regulates TNF-induced early apoptosis. Cells were pretreated with 25 μmol/L honokiol for 12 hours and then incubated with 1 nmol/L TNF for 16 hours. Cells were incubated with anti–Annexin V antibody conjugated with FITC and then analyzed with a flow cytometer for early apoptotic effects.

Honokiol Suppresses RANKL-Induced Osteoclastogenesis

Because RANKL, a member of the TNF superfamily, induces osteoclastogenesis through the activation of NF-κB (18), we investigated whether honokiol can suppress RANKL-induced osteoclastogenesis. We found that RANKL induced osteoclast differentiation as indicated by the expression of tartrate-resistant acid phosphatase and that honokiol suppressed it (Fig. 2A and B). No significant effect of honokiol on the viability of RAW 264.7 cells was observed during this period.

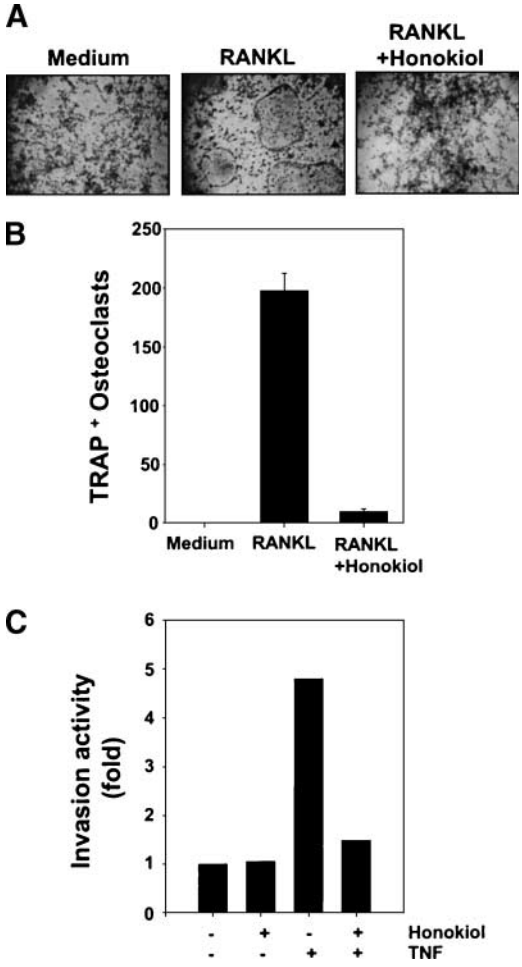


FIGURE 2. Honokiol suppresses RANKL-induced osteoclastogenesis and TNF-induced invasive activity. **A.** RAW 264.7 cells (1×10^4) were plated overnight, pretreated with 5 μmol/L honokiol for 12 hours, and then treated with 5 nmol/L RANKL. At 4 and 5 days later, cells were stained for tartrate-resistant acid phosphatase and evaluated for osteoclastogenesis. Photographs were taken after 5 days of incubation with RANKL. The viability of RAW cells, as determined by MTT method, after treatment with 1, 2.5, and 5 μmol/L honokiol for 5 days was 106%, 108%, and 101%, respectively. **B.** Numbers of tartrate-resistant acid phosphatase–positive multinucleated osteoclasts (>3 nuclei) per well. **C.** H1299 cells (2.5×10^4) were seeded into the upper wells of a Matrigel invasion chamber overnight in the absence of serum, pretreated with 10 μmol/L honokiol for 12 hours, treated with 1 nmol/L TNF for 24 hours in the presence of 1% serum, and then subjected to invasion assay. The value for no honokiol and no TNF was set to 1.0. The viability of H1299 cells, as determined by MTT method, after treatment with honokiol (10 μmol/L), TNF (1 nmol/L), and TNF + honokiol for 24 hours was 92%, 93%, and 91%, respectively.

Honokiol Suppresses TNF-Induced Tumor Cell Invasion Activity

It is known that NF-κB regulates the expression of gene products (e.g., MMP-9) that mediate tumor cell invasion (19). Whether honokiol can modulate TNF-induced tumor cell invasion activity was investigated *in vitro*. To determine this, tumor cells were seeded to the top chamber of the Matrigel invasion chamber with TNF in the presence or absence of 10 μmol/L honokiol and then examined for invasion. As shown in Fig. 2C, TNF induced tumor cell invasion by ~5-fold, and honokiol suppressed this activity. Honokiol alone had no invasion activity. No significant effect of honokiol or of TNF on the viability of H1299 cells was observed during this period.

Honokiol Blocks NF-κB Activation Induced by Various Agents

We investigated whether honokiol modulates NF-κB activation. We first examined the effect of honokiol on the activation of NF-κB induced by various agents, including TNF, phorbol 12-myristate 13-acetate (PMA), okadaic acid, cigarette smoke condensate, and H_2O_2. A DNA-binding assay [electrophoretic mobility shift assay (EMSA)] showed that honokiol suppressed the NF-κB activation induced by all these agents (Fig. 3A). These results suggest that honokiol acted at a step in the NF-κB activation pathway that is common to all these agents.

Honokiol Suppresses NF-κB Activation in a Dose- and Time-Dependent Manner

We next determined the dose and time of exposure to honokiol required to suppress NF-κB activation. The EMSA results showed that honokiol alone had no effect on NF-κB activation. However, it inhibited TNF-mediated NF-κB activation in a dose-dependent manner (Fig. 3B). The suppression of NF-κB activation by honokiol was also found to be time dependent (Fig. 3C).

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

It has been reported that the NF-κB induction pathway in epithelial cells may differ from that in lymphoid cells (20). We therefore investigated whether honokiol inhibited NF-κB activation in different cell types. Honokiol completely inhibited TNF-induced NF-κB activation in embryonic kidney cells (A293) and T-cell leukemia (Jurkat) cells (Fig. 3D), indicating a lack of cell type specificity.

Honokiol Inhibits Constitutive NF-κB Activation

We next tested the effect of honokiol on NF-κB activation in human multiple myeloma (U266) and head and neck squamous cell carcinoma (SCC4) tumor cells, which both express constitutively active NF-κB (21, 22). U266 and SCC4 cells were treated with different concentrations of honokiol for 24 hours and then analyzed NF-κB activation. Honokiol inhibited constitutively active NF-κB in both cells in a dose-dependent manner (Fig. 3E). These results indicated a lack of cell type specificity.

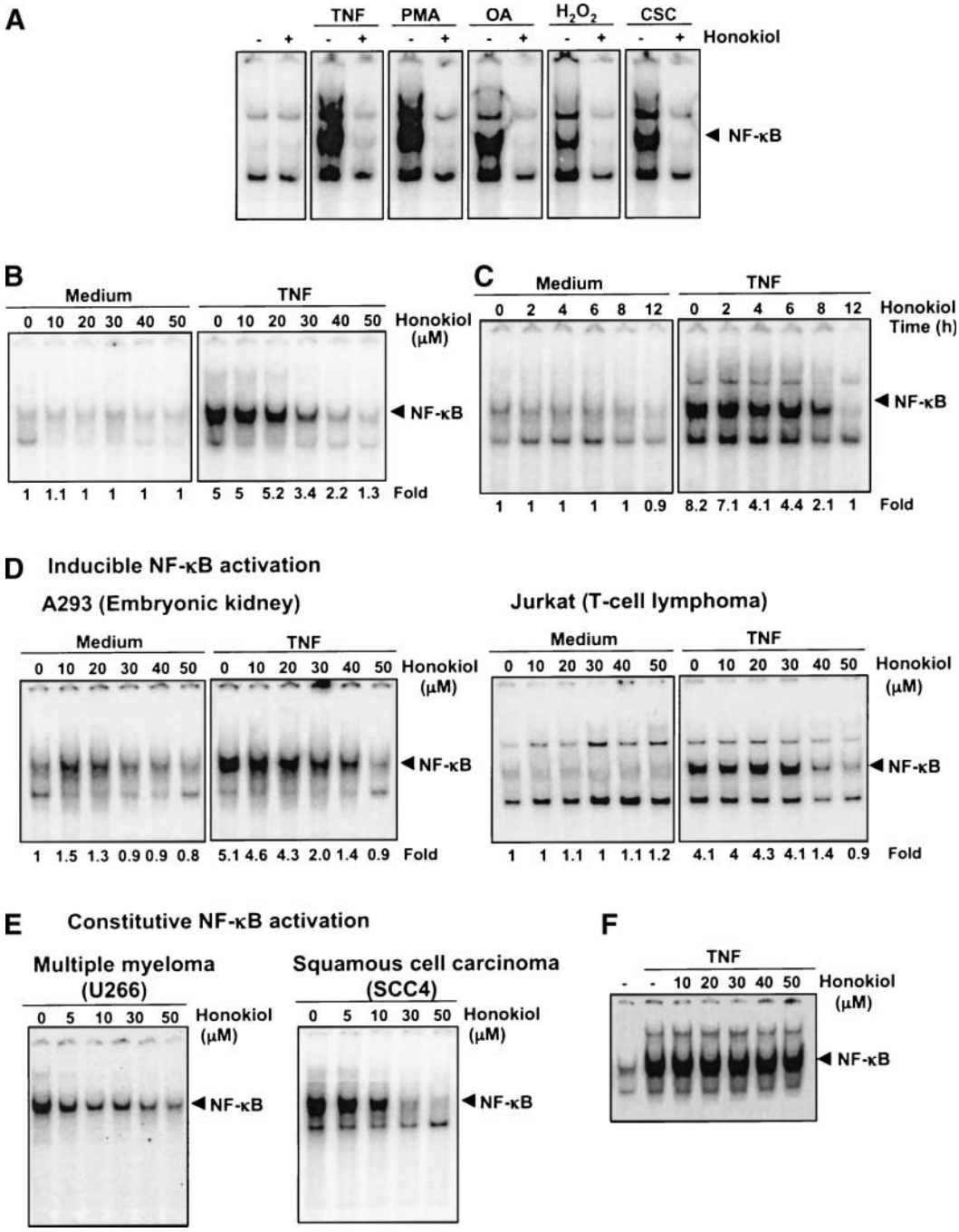


FIGURE 3. Honokiol inhibits NF-κB. **A.** Honokiol blocks NF-κB activation induced by TNF, cigarette smoke condensate (*CSC*), PMA, okadaic acid (*OA*), and H_2O_2. H1299 cells (2×10^6/mL) were preincubated for 12 hours at 37°C with 25 μmol/L honokiol and then treated with TNF (0.1 nmol/L), PMA (25 ng/mL, 1 hour), okadaic acid (500 nmol/L, 4 hours), cigarette smoke condensate (10 μg/mL, 30 minutes), or H_2O_2 (250 μmol/L, 1 hour). Nuclear extracts were prepared and tested for NF-κB activation as described in Materials and Methods. Representative of three independent experiments showing similar results. **B.** Honokiol inhibits TNF-dependent NF-κB activation in a dose-dependent manner. H1299 cells (2×10^6/mL) were preincubated with the indicated concentrations of honokiol for 12 hours at 37°C and then treated with 0.1 nmol/L TNF for 30 minutes. Nuclear extracts were prepared and tested for NF-κB activation as described in Materials and Methods. **C.** Honokiol inhibits TNF-dependent NF-κB activation in a time-dependent manner. H1299 cells (2×10^6/mL) were preincubated with 25 μmol/L honokiol for the indicated times at 37°C and then treated with 0.1 nmol/L TNF for 30 minutes at 37°C. Nuclear extracts were prepared and then tested for NF-κB activation. **D.** Suppression of inducible activation by honokiol is not cell type specific. A293 or Jurkat cells (2×10^6) were pretreated with the indicated concentrations of honokiol for 12 hours and then treated with 0.1 nmol/L TNF for 30 minutes. The nuclear extracts were then prepared and assayed for NF-κB by EMSA as described in Materials and Methods. **E.** Honokiol suppresses constitutive NF-κB activation in multiple myeloma U266 and squamous cell carcinoma SCC4. Cells were incubated with the indicated concentrations of honokiol for 24 hours. Nuclear extracts were prepared and analyzed for NF-κB activation by EMSA. **F.** Honokiol does not modulate the ability of NF-κB to bind to the DNA. Nuclear extracts from H1299 cells (2×10^6/mL) treated or not treated with 0.1 nmol/L TNF for 30 minutes were treated with the indicated concentrations of honokiol for 2 hours at room temperature and then assayed for DNA binding by EMSA. Representative of three independent experiments showing similar results.

Honokiol Does Not Directly Affect Binding of NF-κB to the DNA

Some NF-κB inhibitors, including *N*-tosyl-L-phenylalanine chloromethyl ketone (the serine protease inhibitor), herbimycin A (protein tyrosine kinase inhibitor), and caffeic acid phenethyl ester, directly modify NF-κB to suppress its DNA binding (23-25). We examined whether honokiol mediates its effect through similar mechanism. EMSA result showed that honokiol did not modify the DNA-binding ability of NF-κB proteins prepared from TNF-treated cells (Fig. 3F). These results suggest that honokiol inhibits NF-κB activation by a mechanism different from that of *N*-tosyl-L-phenylalanine chloromethyl ketone, herbimycin A, or caffeic acid phenethyl ester.

Honokiol Inhibits TNF-Dependent IκBα Degradation

To determine the effect of honokiol on time course of TNF-induced NF-κB activation, we pretreated cells with 25 μmol/L honokiol for 12 hours, exposed them to 0.1 nmol/L TNF for different times, and then examined for NF-κB in the nucleus by EMSA. The activation of NF-κB by TNF was found to be time dependent. Treatment of cells with honokiol showed suppression of activation of NF-κB induced by TNF (Fig. 4A). Because IκBα degradation is required for activation of NF-κB (26), we determined whether the inhibition of TNF-induced NF-κB activation by honokiol was due to inhibition of IκBα degradation. We found that TNF induced IκBα degradation in control cells as early as 10 minutes, but in honokiol-pretreated cells TNF had no effect on IκBα degradation (Fig. 4B).

Honokiol Inhibits TNF-Dependent IκBα Phosphorylation

We next determined whether honokiol affected the TNF-induced IκBα phosphorylation needed for IκBα degradation. We used *N*-acetyl-leucyl-leucyl-norleucinal, which prevents the degradation of phosphorylated IκBα. Western blot analysis using antibody that detects only the serine-phosphorylated form of IκBα indicated that TNF induced IκBα phosphorylation and that honokiol completely suppressed it (Fig. 4C). Thus, honokiol inhibited TNF-induced NF-κB activation by inhibiting phosphorylation and degradation of IκBα.

Honokiol Inhibits TNF-Dependent Ubiquitination of IκBα

We next determined whether honokiol affected the TNF-induced IκBα ubiquitination that leads to IκBα degradation. Western blot analysis using antibody that detects IκBα indicated that TNF induced IκBα ubiquitination, as indicated by high molecular weight bands, and honokiol completely suppressed it (Fig. 4D). Thus, honokiol inhibited TNF-induced NF-κB activation by inhibiting phosphorylation, ubiquitination, and degradation of IκBα.

Honokiol Inhibits TNF-Induced IKK Activation

Because honokiol inhibits the phosphorylation of IκBα, we tested the effect of honokiol on TNF-induced IKK activation, which is required for TNF-induced phosphorylation of IκBα. As shown in Fig. 4E (*top*), honokiol completely suppressed TNF-induced activation of IKK. TNF or honokiol had no direct effect on the expression of IKK protein (*bottom*).

Next, we examined the effect of honokiol on IKK activity *in vitro*. We found that honokiol did not directly interfere with the IKK activity (data not shown). Because treatment of cells inhibits TNF-induced IKK activity, honokiol must suppress the activation of IKK.

Honokiol Inhibits TNF-Induced Akt Activation

It has been reported that Akt can activate IKK (27). Thus, it is possible that honokiol suppresses TNF-induced Akt activation. To examine the effect of honokiol on the TNF-induced activation of Akt, we pretreated cells with honokiol and then exposed to TNF, prepared whole-cell extracts, and did Western blot analysis using antibody against the Ser^{473}-phosphorylated form of Akt. TNF induced the Akt activation in a time-dependent manner, and honokiol suppressed it (Fig. 4F).

Besides autophosphorylation, activated Akt activation is known to phosphorylate several proteins, including glycogen synthase kinase-3 (GSK-3). Whether honokiol modulates the Akt-induced phosphorylation of GSK-3β was also examined. As shown in Fig. 4G, TNF treatment induced phosphorylation of GSK-3β and honokiol suppressed this phosphorylation.

Honokiol Inhibits TNF-Induced Nuclear Translocation of p65 and Phosphorylation of p65 in the Nuclei

We also tested by Western blot analysis the effect of honokiol on TNF-induced nuclear translocation of p65 and phosphorylation of p65. As shown in Fig. 4H, honokiol suppressed nuclear translocation and phosphorylation of the p65 subunit of NF-κB.

Honokiol Inhibits TNF-Induced Phosphorylation of p65 in the Cytoplasm

Since nuclear translocation of p65 requires its phosphorylation in the cytoplasm, we also tested the effect of honokiol on TNF-induced phosphorylation of p65 (28). As shown in Fig. 4I, honokiol suppressed p65 phosphorylation almost completely. Similarly, immunocytochemical analysis (Fig. 4J) indicated that honokiol abolished TNF-induced nuclear translocation of p65.

Honokiol Represses TNF-Induced NF-κB-Dependent Reporter Gene Expression

Our results up to this point showed that honokiol inhibited the translocation of p65 inside the nucleus. To further show that honokiol inhibited NF-κB-dependent gene transcription, we transiently transfected cells with the NF-κB-regulated secreted alkaline phosphatase (SEAP) reporter construct and then stimulated them with TNF. We found that TNF produced an ~5-fold increase in SEAP activity over vector control (Fig. 5A), which was inhibited by dominant-negative IκBα, indicating specificity. When the cells were pretreated with honokiol, TNF-induced NF-κB-dependent SEAP expression was inhibited in a dose-dependent manner. These results showed that honokiol inhibits the NF-κB-dependent reporter gene expression induced by TNF.

We next determined where honokiol acts in the sequence of TNFR1, TRADD, TRAF2, NIK, and IKK recruitment that characterizes TNF-induced NF-κB activation (29). In cells

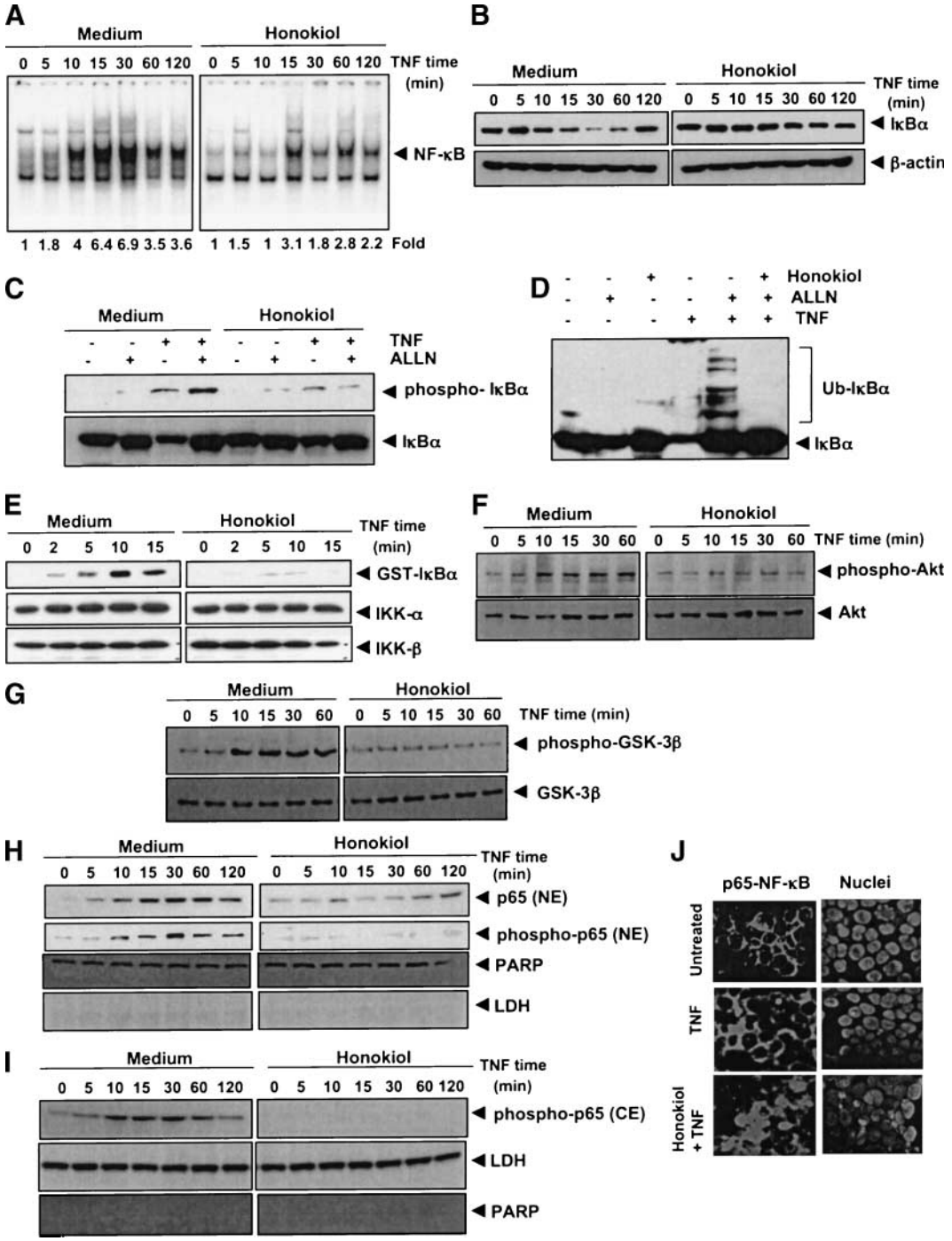
transfected with TNFR1, TRADD, TRAF2, NIK, IKKβ, and p65 plasmids, NF-κB-dependent reporter gene expression was induced; honokiol suppressed SEAP expression in all cells, except those transfected with p65 (Fig. 5B).

Honokiol Represses TNF-Induced COX-2 Promoter Activity

We next determined whether honokiol affected COX-2 promoter activity, which is regulated by NF-κB (30). As shown in Fig. 5C, honokiol inhibited the TNF-induced COX-2 promoter activity in a dose-dependent manner.

Magnolol Also Suppresses NF-κB Activation in a Dose-Dependent Manner

Magnolol is a close structural homologue of honokiol (see Fig. 5D); hence, we investigated whether it can also suppress TNF-induced NF-κB activation. We determined the dose of magnolol required to suppress NF-κB activation. EMSA results



showed that magnolol alone had no effect on NF-κB activation. However, it inhibited TNF-mediated NF-κB activation in a dose-dependent manner (Fig. 5E). The suppression of NF-κB activation by magnolol was comparable with that of honokiol (Fig. 5E).

Honokiol Inhibits TNF-Induced COX-2, MMP-9, Intercellular Adhesion Molecule-1, and VEGF Expression

The above results indicated that honokiol inhibits TNF-induced tumor cell invasion. We investigated whether these effects of honokiol are mediated through the suppression of COX-2, MMP-9, intercellular adhesion molecule-1 (ICAM-1), and VEGF gene products. We found that TNF treatment induced the expression of VEGF, COX-2, ICAM-1, and MMP-9 gene products and that honokiol abolished the expression (Fig. 6A).

Honokiol Inhibits TNF-Induced Cyclin D1 and c-myc Expression

Both cyclin D1 and c-myc regulate cellular proliferation and are regulated by NF-κB (11). Whether honokiol controls the expression of these gene products was also examined. We found that honokiol abolished, in a time-dependent fashion, the TNF-induced expression of cyclin D1 and c-myc (Fig. 6B).

Honokiol Inhibits TNF-Induced Activation of Antiapoptotic Gene Products

The above results indicated that honokiol potentiates the apoptosis induced by TNF. Whether this effect of honokiol is through suppression of antiapoptotic gene products was investigated. NF-κB up-regulates the expression of several genes implicated in facilitating tumor cell survival, including *cIAP1, cIAP2, Bcl-2, Bcl-x_L, cFLIP, TRAF1,* and *survivin* (11). We found that honokiol inhibited the TNF-induced expression of all of these proteins (Fig. 6C).

Honokiol Inhibits NF-κB Activation in Mouse Skin

To determine whether honokiol could suppress NF-κB activation *in vivo*, shaved mouse skin was pretreated for 1 hour with honokiol and then stimulated with PMA for 4 hours (Fig. 7A). Nuclear extracts were prepared and assayed for NF-κB by EMSA. As shown in Fig. 7B, none of the untreated skin samples expressed NF-κB DNA-binding activity; tissues derived from four of five PMA-treated animals expressed NF-κB DNA-binding activity compared with untreated group. The trend indicated that honokiol pretreatment suppressed PMA-induced NF-κB DNA-binding activity.

Discussion

The present study was designed to investigate the effect of honokiol on the NF-κB activation pathway and on the NF-κB-regulated gene products that control tumor cell survival, proliferation, invasion, angiogenesis, and metastasis (see Fig. 7C). We found that honokiol potentiated the apoptosis induced by TNF and chemotherapeutic agents and inhibited TNF-induced invasion and RANKL-induced osteoclastogenesis. Honokiol suppressed NF-κB activated by carcinogens, tumor promoters, and inflammatory stimuli in a variety of cell lines. This inhibition was mediated through inhibition of Akt and IKK activation by honokiol, which led to suppression of phosphorylation and degradation of IκBα. Honokiol also inhibited the TNF-induced phosphorylation of p65, nuclear p65 translocation, and NF-κB-dependent reporter gene activity. The expressions of gene products involved in antiapoptosis (IAP1, IAP2, survivin, Bcl-2, Bcl-x_L, TRAF1, and cFLIP), proliferation (cyclin D1 and c-myc), and metastasis (MMP-9, COX-2, and VEGF) were also down-regulated by honokiol. Honokiol also suppressed PMA-induced NF-κB activation in mouse skin *in vivo*.

Our results indicate that honokiol suppresses NF-κB activated by a variety of agents. This is the first report to examine the effect of honokiol on NF-κB activated by various

FIGURE 4. **A.** Honokiol inhibits TNF-induced NF-κB activation, IκBα phosphorylation, and IκBα degradation. Honokiol inhibits TNF-induced activation of NF-κB. H1299 cells were incubated with 25 μmol/L honokiol for 12 hours, treated with 0.1 nmol/L TNF for the indicated times, and then analyzed for NF-κB activation by EMSA. **B.** H1299 cells (2×10^6/mL) were incubated with 25 μmol/L honokiol for 12 hours at 37°C, treated with 0.1 nmol/L TNF for the indicated times at 37°C, and then tested for IκBα (*top*) in cytosolic fractions by Western blot analysis. Equal protein loading was evaluated by β-actin (*bottom*). Honokiol blocks the phosphorylation (**C**) and ubiquitination (**D**) of IκBα by TNF. Cells were preincubated with 25 μmol/L honokiol for 12 hours, incubated with 50 μg/mL *N*-acetyl-leucyl-leucyl-norleucinal (*ALLN*) for 30 minutes and then treated with 0.1 nmol/L TNF for 10 minutes. Cytoplasmic extracts were fractionated and then subjected to Western blot analysis using phosphospecific anti-IκBα antibody. The same membrane was reblotted with anti-IκBα antibody. **E.** Honokiol inhibits TNF-induced IKK activity. H1299 cells (2×10^6/mL) were treated with 25 μmol/L honokiol for 12 hours and then treated with 0.1 nmol/L TNF for the indicated time intervals. Whole-cell extracts were prepared, and 200 μg extract was immunoprecipitated with antibodies against IKKα and IKKβ. The immunocomplex kinase assay was then done as described in Materials and Methods. To examine the effect of honokiol on the level of expression of IKK proteins, 30 μg whole-cell extract was analyzed on 10% SDS-PAGE, electrotransferred, and immunoblotted with the indicated antibodies as described in Materials and Methods. **F.** Honokiol inhibits TNF-induced activation of Akt. H1299 cells (2×10^6/mL) were treated with 25 μmol/L honokiol for 12 hours and then treated with 0.1 nmol/L TNF for the indicated time intervals. Whole-cell extracts were prepared and Western blot analysis was done using anti-phosphospecific Akt (Ser^{473}) antibody. The same blot was reprobed with nonphosphorylated Akt antibody. **G.** Honokiol suppresses Akt-mediated phosphorylation of GSK-3β. H1299 cells (2×10^6/mL) were treated with 25 μmol/L honokiol for 12 hours and then treated with 0.1 nmol/L TNF for the indicated time intervals. Whole-cell extracts were prepared and Western blot analysis was done using anti-phosphospecific GSK-3β (Ser^9) antibody. The same blot was reprobed with nonphosphorylated GSK-3β antibody. **H.** Honokiol inhibits TNF-induced nuclear translocation of p65 and phosphorylation of p65 in the nuclei. H1299 cells (2×10^6/mL) were either untreated or pretreated with 25 μmol/L honokiol for 12 hours at 37°C and then treated with 0.1 nmol/L TNF for the indicated times. Nuclear extracts were prepared and analyzed by Western blotting using antibodies against p65 and phosphorylated form of p65. To examine the purity of the nuclear extracts (*NE*), gels were stripped and reprobed with antibodies against lactate dehydrogenase (*LDH*; cytoplasmic marker) and against PARP (nuclear marker). **I.** Honokiol inhibits TNF-induced phosphorylation of p65 in the cytoplasm. H1299 cells (2×10^6/mL) were incubated with 25 μmol/L honokiol for 12 hours and then treated with 0.1 nmol/L TNF for the indicated times. The cytoplasmic extracts (*CE*) were analyzed by Western blotting using antibodies against the phosphorylated form of p65. To examine the purity of the cytoplasmic extracts, gels were stripped and reprobed with antibodies against lactate dehydrogenase (cytoplasmic marker) and against PARP (nuclear marker). **J.** Honokiol inhibits TNF-induced nuclear translocation of p65. H1299 cells (1×10^6/mL) were first treated with 25 μmol/L honokiol for 12 hours at 37°C and then exposed to 0.1 nmol/L TNF. After fixation, immunocytochemical analysis was done as described in Materials and Methods. Representative of three independent experiments showing similar results.

stimuli. These results suggest that honokiol must act at a step common to all these agents. We found that honokiol blocked the activation of NF-κB without directly interfering with the DNA binding of NF-κB. Further analysis of the pathway indicated that honokiol targets at the level of IKK. However, our *in vitro* kinase assay results showed that honokiol is not a direct inhibitor of IKK. Thus, it seems that honokiol blocks the activation of IKK by interfering with some upstream regulatory kinases. Akt, NIK, mitogen-activated protein kinase kinase kinase 1, and atypical protein kinase C are candidates because they are upstream kinases that regulate IKK (11). Indeed, our results show that honokiol suppresses TNF-induced activation of Akt.

We found that honokiol inhibited not only inducible NF-κB activation but also constitutively activated NF-κB in multiple

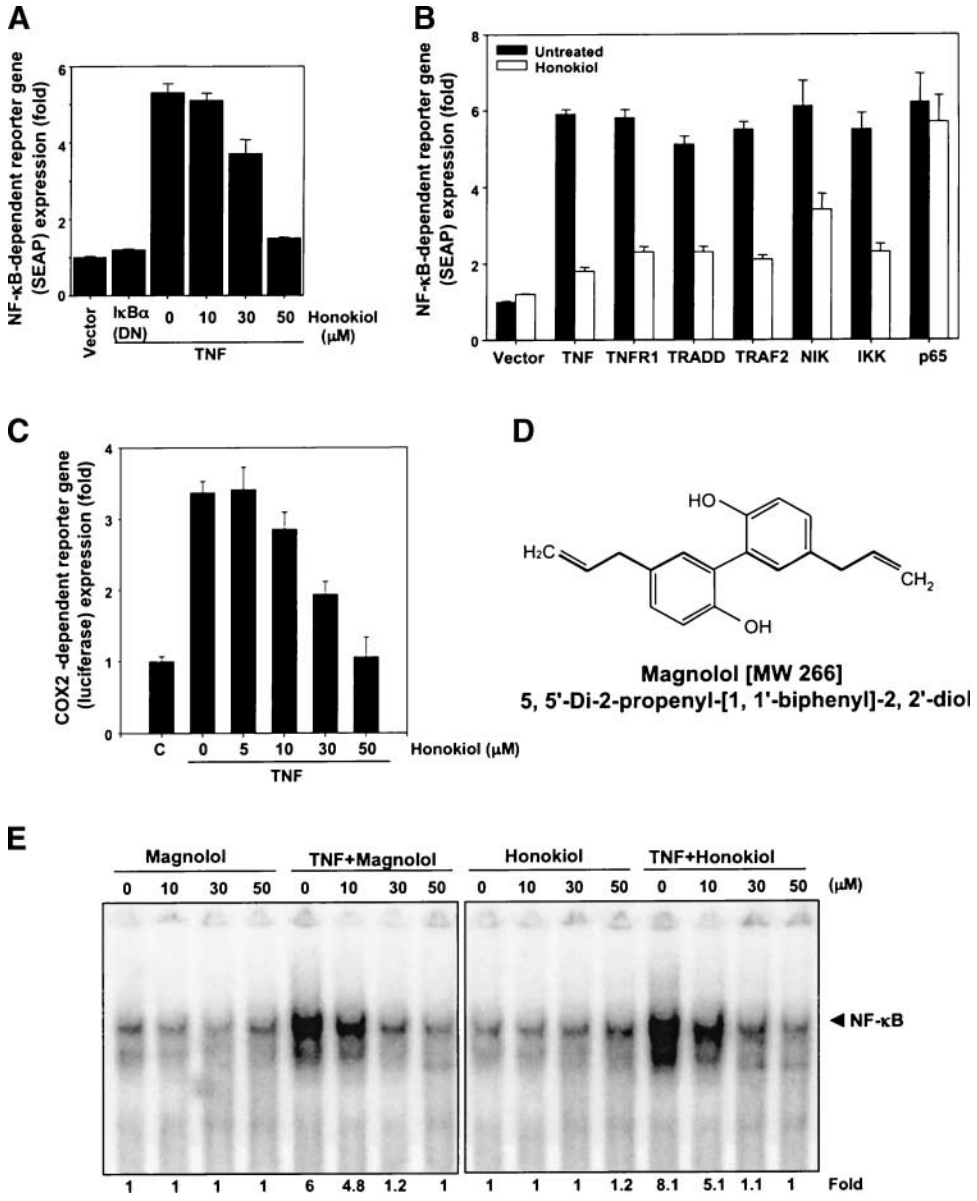


FIGURE 5. **A.** Honokiol inhibits TNF-induced NF-κB-dependent reporter gene (SEAP) expression. A293 cells were transiently transfected with a NF-κB-containing plasmid linked to the SEAP gene and then treated with the indicated concentrations of honokiol. After 24 hours in culture with 1 nmol/L TNF, 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.** Honokiol inhibits NF-κB-dependent reporter gene expression induced by TNFR1, TRADD, TRAF, NIK, and IKKβ. A293 cells were transiently transfected with the indicated plasmids along with a NF-κB-containing plasmid linked to the SEAP gene and then left either untreated or treated with 25 μmol/L honokiol for 12 hours. 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. **C.** Honokiol inhibits TNF-induced COX-2 promoter activity. H1299 cells were transiently transfected with a COX-2 promoter plasmid linked to the luciferase gene and then treated with the indicated concentrations of honokiol. After 24 hours in culture with 1 nmol/L TNF, cell supernatants were collected and assayed for luciferase activity as described in Materials and Methods. Results are expressed as fold activity over the activity of the vector control. Representative of three independent experiments showing similar results. Columns, mean of triplicate cultures; bars, SD. **D.** Structure of magnolol. **E.** The honokiol analogue magnolol inhibits TNF-induced NF-κB activation. H1299 cells were treated with the indicated concentrations of magnolol for 12 hours and then stimulated with 0.1 nmol/L TNF for 30 minutes. Nuclear extracts were prepared and analyzed for NF-κB activation by EMSA.

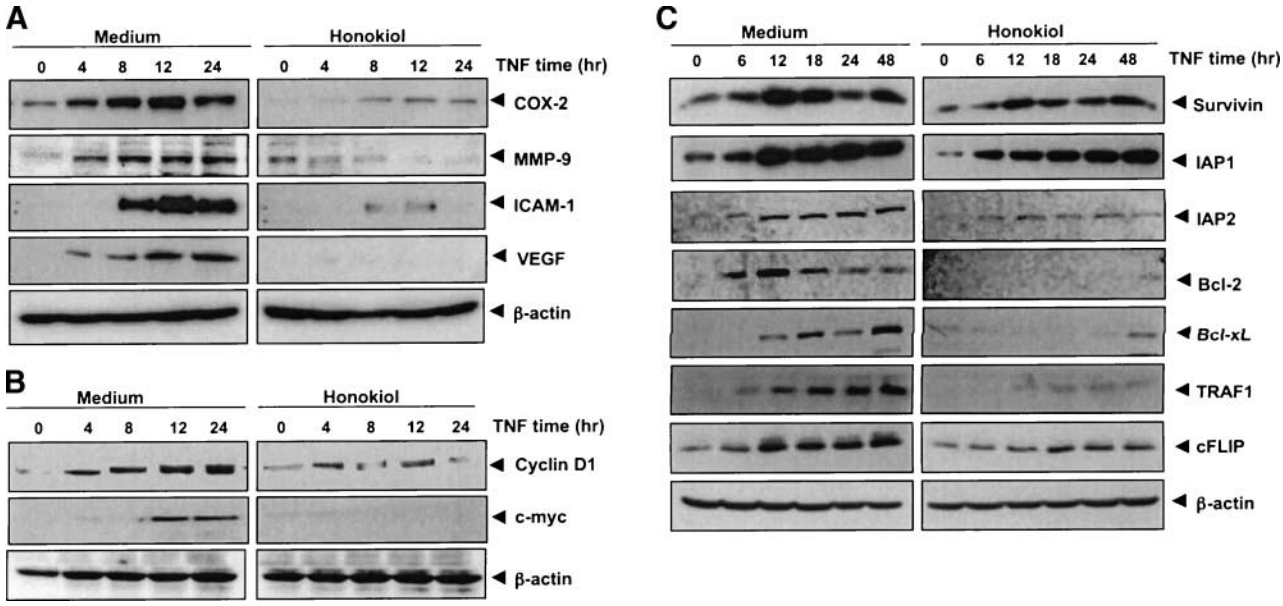


FIGURE 6. Honokiol inhibits TNF-induced NF-κB-regulated gene products. **A.** Honokiol inhibits COX-2, MMP-9, ICAM-1, and VEGF expression induced by TNF. H1299 cells (2×10^6/mL) were left untreated or incubated with 25 μmol/L honokiol for 12 hours and then treated with 1 nmol/L TNF for different times. Whole-cell extracts were prepared, and 50 μg whole-cell lysate was resolved by SDS-PAGE, electrotransferred to nitrocellulose membrane, sliced based on the molecular weight, and then probed with antibodies against VEGF, MMP-9, ICAM-1, COX-2, or β-actin as described in Materials and Methods. TNF-treated and TNF + honokiol–treated samples were run on the same gel under identical conditions and probed with the same immunoblotting solutions. **B.** Honokiol inhibits cyclin D1 and c-myc expression induced by TNF. H1299 cells (2×10^6/mL) were left untreated or incubated with 25 μmol/L honokiol for 12 hours and then treated with 1 nmol/L TNF for different times. Whole-cell extracts were prepared, and 50 μg whole-cell lysate was analyzed by Western blotting using antibodies against cyclin D1 and c-myc. Representative of three independent experiments showing similar results. TNF-treated and TNF + honokiol–treated samples were run on the same gel under identical conditions and probed with the same immunoblotting solutions. **C.** Honokiol inhibits the expression of antiapoptotic gene products cIAP1, cIAP2, Bcl-x_L, Bcl-2, cFLIP, TRAF2, and survivin. H1299 cells (2×10^6/mL) were left untreated or incubated with 25 μmol/L honokiol for 12 hours and then treated with 1 nmol/L TNF for different times. Whole-cell extracts were prepared, and 50 μg whole-cell lysate was analyzed by Western blotting using antibodies against IAP1, IAP2, Bcl-x_L, Bcl-2, cFLIP, and survivin. TNF-treated and TNF + honokiol–treated samples were run on the same gel under identical conditions and probed with the same immunoblotting solutions. Representative of three independent experiments.

myeloma and head and neck squamous cell carcinoma cells. Constitutive active NF-κB activation has been found to be critical for the survival and proliferation of various tumor cell types (11); however, the mechanism of constitutive NF-κB activation is not well understood. Some of the potential mechanisms are overexpression of IκBα without inhibition of NF-κB activity, mutations in the IκBα gene, enhanced IκBα degradation, and constitutive expression of TNF and interleukin-1 (11).

We found that honokiol suppresses NF-κB-dependent reporter gene expression. Honokiol also inhibited the TNF-induced COX-2 promoter activity, which is regulated by NF-κB (30). These results agree with a recent report (5) showing that honokiol inhibits NF-κB luciferase reporter activity. We also found that honokiol suppressed NF-κB activation induced by overexpression of TNFR1, TRADD, TRAF2, NIK, and IKK plasmids but had no effect on activation induced by p65 plasmid. These results suggest that honokiol acts at a step between IKK and p65.

The genes that are involved in the proliferation and metastasis of cancer have been shown to be regulated by NF-κB (11). We showed in this report that honokiol inhibits the expression of cyclin D1 and c-myc, both regulated by NF-κB. Our results also showed that the expressions of COX-2, MMP-9, ICAM-1, and VEGF, which are also regulated by NF-κB, are down-regulated by honokiol. Indeed, honokiol has been shown to down-regulate COX-2 gene expression in the human monocytic THP1 cell line (31). The suppression of invasion (32) and angiogenesis (33) reported previously agrees with the results reported here. The inflammatory cytokines TNF and interleukin-8, both known to promote angiogenesis, have also been shown to be down-regulated by honokiol (4, 5). The down-regulation of nitric oxide synthesis by honokiol reported previously (4) also likely occurs through the suppression of NF-κB activation as reported here.

We also found for the first time that honokiol suppressed RANKL-induced osteoclastogenesis. RANKL mediates osteoclastogenesis in part through activation of NF-κB (34). Thus, it is very likely that suppression of NF-κB by honokiol leads to suppression of osteoclastogenesis.

NF-κB is known to regulate the expression of IAP1, IAP2, Bcl-x_L, Bcl-2, TRAF1, and cFLIP, and their overexpression in numerous tumors has been linked to survival, chemoresistance, and radioresistance. Our results indicated that honokiol treatment down-regulates the TNF-induced expression of these gene products. Earlier studies have shown that honokiol down-regulates the expression of the antiapoptotic proteins Bcl-x_L (7) and Mcl-1 (35). Our studies also showed that honokiol potentiated the apoptotic effects of TNF, paclitaxel, and doxorubicin. These effects are similar to that reported with a specific inhibitor of NF-κB (36). Two recent reports (10, 35) that honokiol induces apoptosis of human B-cell chronic lymphocytic leukemia and human multiple myeloma through the activation of caspases also agree with the results shown here. Overall, our results indicate that the antiproliferative,

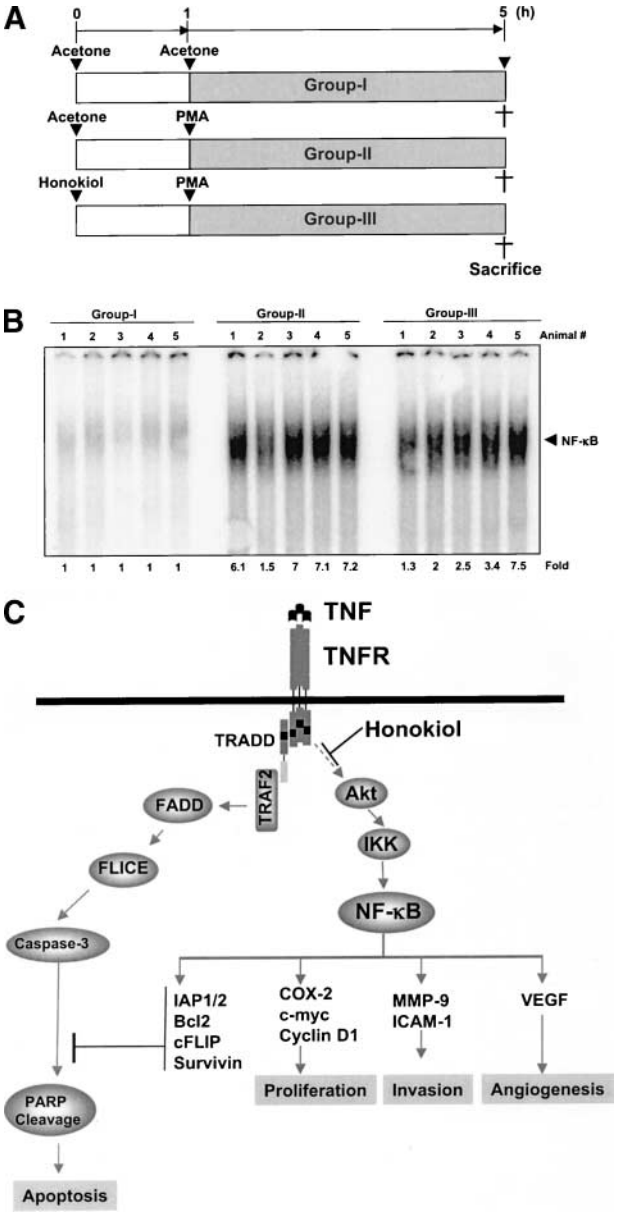


FIGURE 7. Honokiol inhibits NF-κB activation in mouse skin model **A.** Brief description of animal experiment. **B.** Honokiol inhibits PMA-induced NF-κB activation in mice dorsal skin. Shaved mouse skin was pretreated 200 μL honokiol (1.3 mmol/L) and then treated with 20 μL PMA (5 μg/mL). After 4 hours, mice were sacrificed, and skin biopsies were taken. Nuclear extracts were prepared and assayed for NF-κB by EMSA. **C.** Schematic representation of the effect of honokiol on TNF-induced NF-κB activation and apoptosis.

proapoptotic, anti-invasive, antiosteoclastogenic, antiangiogenic, and antimetastatic effects of honokiol may be mediated through suppression of NF-κB-regulated gene products.

Materials and Methods

Materials

Honokiol and magnolol were isolated in our laboratory as described previously (8). A 50 mmol/L solution of honokiol and magnolol 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 5×10^7 units/mg, was kindly provided by Genentech (South San Francisco, CA). Cigarette smoke condensate, prepared as described previously (37), was kindly supplied by Dr. C.G. Gairola (University of Kentucky, Lexington, KY). Penicillin, streptomycin, Iscove's modified Dulbecco's medium, and fetal bovine serum were obtained from Invitrogen (Grand Island, NY). PMA, okadaic acid, H_2O_2, and anti-β-actin antibody were obtained from Sigma-Aldrich (St. Louis, MO). Antibodies against p65, p50, IκBα, cyclin D1, MMP-9, PARP, IAP1, IAP2, Bcl-2, Bcl-x_L, VEGF, c-myc, ICAM-1, Akt, and lactate dehydrogenase and the Annexin V staining kit were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-COX-2 and anti-XIAP antibodies were obtained from BD Biosciences (San Diego, CA). Phosphospecific anti-IκBα (Ser^{32}), phosphospecific anti-p65 (Ser^{536}), GSK-3β, and phosphospecific GSK-3β (Ser^{9}) antibodies were purchased from Cell Signaling (Beverly, MA). Anti-IKKα, anti-IKKβ, phosphorylated Akt (Ser^{473}), and anti-FLIP antibodies were kindly provided by Imgenex (San Diego, CA). All these antibodies have been shown to be specific towards the antigen employed.

Cell Lines

Human myeloid KBM-5 cells, mouse macrophage RAW 264.7 cells, human lung adenocarcinoma H1299 cells, human multiple myeloma U266 cells, squamous cell carcinoma SCC4 cells, and human embryonic kidney A293 cells were obtained from American Type Culture Collection (Manassas, VA). KBM-5 cells were cultured in Iscove's modified Dulbecco's medium supplemented with 15% fetal bovine serum. RAW 264.7 cells were cultured in DMEM/F-12, H1299 cells and U266 were cultured in RPMI 1640, and A293 cells were cultured in DMEM supplemented with 10% fetal bovine serum. SCC4 cells were cultured in DMEM containing 10% fetal bovine serum, nonessential amino acids, pyruvate, glutamine, and vitamins. All media were also supplemented with 100 units/mL penicillin and 100 μg/mL streptomycin.

Cytotoxicity Assay

Cytotoxicity was assayed by the modified MTT assay as described previously (38).

PARP Cleavage Assay

For detection of cleavage products of PARP, whole-cell extracts were prepared by subjecting TNF and honokiol-treated cells to 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 μg/mL aprotinin, 0.005 μg/mL leupeptin, 0.4 mmol/L phenylmethylsulfonyl fluoride, 4 mmol/L $NaVO_4$]. Lysates were spun at 14,000 rpm for 10 minutes to remove insoluble material, resolved by 10% SDS-PAGE, and probed with PARP antibodies.

Live/Dead Assay

To measure apoptosis, we used the Live/Dead assay (Molecular Probes, Eugene, OR), which determines intracellular

esterase activity and plasma membrane integrity. This assay uses calcein-AM, a polyanionic dye, which is retained within live cells and provides green fluorescence (38). 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, 1×10^5 cells were incubated with 10 μmol/L honokiol for 24 hours and then treated with 1 nmol/L TNF for 16 hours at 37°C. Cells were stained with the Live/Dead reagent (5 μmol/L ethidium homodimer, 5 μmol/L calcein-AM) and then incubated at 37°C for 30 minutes. Cells were analyzed under a fluorescence microscope (Labophot-2, Nikon, Tokyo, Japan).

Annexin V Assay

One of the early indicators of apoptosis is the rapid translocation and accumulation of the membrane phospholipid phosphatidylserine from the cytoplasmic interface to the extracellular surface of the cell. This loss of membrane asymmetry can be detected using the binding properties of Annexin V. To detect apoptosis, we employed Annexin V antibody conjugated with the fluorescent dye FITC. Briefly, 1×10^6 cells were pretreated with 30 μmol/L honokiol for 12 hours, treated with 1 nmol/L TNF for 16 hours, and then subjected to Annexin V staining. Cells were washed, stained with FITC-conjugated anti–Annexin V antibody, and then analyzed with a flow cytometer (FACSCalibur, BD Biosciences).

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 BioCoat Tumor Invasion system is a chamber that has a light-tight polyethylene terephthalate membrane with 8-μm-diameter pores and is coated with a reconstituted basement membrane gel (BD Biosciences). A total of 2.5×10^4 H1299 cells were suspended in serum-free medium and seeded into the upper wells. After incubation overnight, cells were treated with 10 μmol/L honokiol for 12 hours and then stimulated with 1 nmol/L TNF for a further 24 hours in the presence of 1% fetal bovine serum and the honokiol. The cells that invaded through the Matrigel (i.e., those that migrated to the lower chamber during incubation) were stained with 4 μg/mL calcein-AM (Molecular Probes) in PBS for 30 minutes at 37°C and scanned for fluorescence with a Victor 3 multiplate reader (Perkin-Elmer Life and Analytical Sciences, Boston, MA); fluorescent cells were counted.

Osteoclast Differentiation Assay

To determine the effect of honokiol on RANKL-induced osteoclastogenesis, we cultured RAW 264.7 cells, which can differentiate into osteoclasts by RANKL *in vitro* (38). RAW 264.7 cells were cultured in 24-well dishes at a density of 1×10^4 per well and allowed to adhere overnight. The medium was then replaced, and the cells were pretreated with 5 μmol/L honokiol for 12 hours and then treated with 5 nmol/L RANKL. At days 4 and 5, the cells were stained for tartrate-resistant acid phosphatase expression as described previously (39) using an acid phosphatase kit (Sigma-Aldrich), and the tartrate-resistant acid phosphatase–positive multinucleated osteoclasts (>3 nuclei) per well were counted.

Electrophoretic Mobility Shift Assay

To determine NF-κB activation by TNF, which has a well-established role in inflammation, tumor proliferation, promotion, invasion, and metastasis (13), we did EMSA essentially as described previously (40). Briefly, nuclear extracts prepared from TNF-treated cells (1×10^6/mL) 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**GGGGACTTTC**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′-TTGTTACAA-**CTCACTTTC**CGCTG**CTCACTTTC**CAGGGA-GGCGTGG-3′, was used to examine the specificity of binding of NF-κB 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 the p50 or the p65 subunit of NF-κB for 30 minutes at 37°C before the complex was analyzed by EMSA. Preimmune serum was included as a negative control. The dried gels were visualized with a Storm820 and radioactive bands were quantified using ImageQuant software (Amersham Pharmacia Biotechnology, Piscataway, NJ).

Western Blot Analysis

To determine the effect of honokiol on TNF-dependent IκBα phosphorylation, IκBα degradation, p65 translocation, and p65 phosphorylation, cytoplasmic extracts were prepared as described previously (41) from H1299 cells (2×10^6/mL) that had been pretreated with 25 μmol/L honokiol for 12 hours and then exposed to 0.1 nmol/L TNF for various times. Cytoplasmic protein (30 μg) was resolved on 10% SDS-PAGE gel, transferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with specific antibodies against IκBα, phosphorylated IκBα, p65, and phosphorylated p65. To determine the effect of honokiol on TNF-induced expression of cyclin D1, COX-2, MMP-9, cIAP1, cIAP2, TRAF1, ICAM-1, c-myc, Bcl-2, Bcl-x_L, VEGF, cFLIP, and survivin in whole-cell extracts of treated cells (2×10^6 in 1 mL medium), 50 μg protein was resolved on SDS-PAGE and probed by Western blot with specific antibodies according to the manufacturer's recommended protocol. Whole-cell lysate was resolved by SDS-PAGE, electrotransferred to nitrocellulose membrane, sliced based on the molecular weight, and then probed with antibodies against various proteins. The blots were washed, exposed to horseradish peroxidase–conjugated secondary antibodies for 1 hour, and finally detected by enhanced chemiluminescence reagent (Amersham Pharmacia Biotechnology). The bands were quantified using a Personal Densitometer Scan version 1.30 using ImageQuant software version 3.3 (Molecular Dynamics, Sunnyvale, CA).

IKK Assay

To determine the effect of honokiol on TNF-induced IKK activation, we analyzed IKK by a method essentially as described previously (41). Briefly, the IKK complex from whole-cell extracts was precipitated with antibodies against IKKα and IKKβ and then treated with protein A/G-Sepharose beads (Pierce Chemical, Rockford, IL). After 2 hours, 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 μCi [γ-^{32}P]ATP, 10 μmol/L unlabeled ATP, and 2 μg substrate glutathione *S*-transferase-IκBα (amino acids 1-54). After incubation at 30°C for 30 minutes, the reaction was terminated by boiling with SDS sample buffer for 5 minutes. Finally, the protein was resolved on 10% SDS-PAGE, the gel was dried, and the radioactive bands were visualized with a Storm820. To determine the total amounts of IKKα and IKKβ 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-IKKα or anti-IKKβ antibody.

Immunolocalization of NF-κB p65

The effect of honokiol on the TNF-induced nuclear translocation of p65 was examined by an immunocytochemical method using an epifluorescence microscope (Labophot-2, Nikon, Tokyo, Japan) and a Photometrics Coolsnap CF color camera (Nikon, Lewisville, TX) as described previously (41).

NF-κB-Dependent Reporter Gene Transcription

The effect of honokiol on TNF-induced NF-κB dependent reporter gene transcription in A293 cells was measured as described previously (41).

COX-2 Promoter-Dependent Reporter Luciferase Gene Expression

COX-2 promoter activity was examined as described elsewhere (41). To further determine the effect of honokiol on COX-2 promoter, A293 cells were seeded at a concentration of 1.5×10^5 per well in six-well plates. After overnight culture, the cells in each well were transfected with 2 μg DNA consisting of COX-2 promoter-luciferase reporter plasmid along with 6 μL LipofectAMINE 2000 according to the manufacturer's protocol. The COX-2 promoter (−375 to +59), which was amplified from human genomic DNA by using the primers 5′-GAGTCTCTTATTTATTTTT-3′ (sense) and 5′-GCTGCTGAGGAGTTCCTGGACGTGC-3′ (antisense), was kindly provided by Dr. Xiao-Chun Xu (M. D. Anderson Cancer Center). After a 6-hour exposure to the transfection mixture, the cells were incubated in medium containing honokiol for 12 hours. The cells were exposed to TNF (0.1 nmol/L) for 24 hours and then harvested. Luciferase activity was measured by using the Luclite luciferase assay system (Perkin-Elmer) according to the manufacturer's protocol and detected by luminometer (Victor 3). All experiments were done in triplicate and repeated at least twice to prove their reproducibility.

Animal Experiment

Female ICR mice (6-7 weeks old) were purchased from Harlan Sprague-Dawley, Inc. (Indianapolis, IN) The animals were quarantined for 2 days and were housed in climate-controlled quarters (24 ± 1°C at 50% humidity) with a 12-hour light/12-hour dark cycle. The dorsal side of skin was shaved using an electric clipper, and only those animals in the resting phase of the cycle were used in all experiments. Honokiol and PMA were dissolved in acetone and applied to the dorsal shaven area. Shaved mouse skin was pretreated with 200 μL honokiol (1.3 mmol/L) and then after 1 hour was applied with 20 μL PMA (5 μg/mL). After 4 hours, mice were sacrificed and skin biopsies were taken. Skin tissue was minced and incubated on ice for 30 minutes ice-cold buffer A [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L KCl, 10 mmol/L $MgCl_2$, 0.5 mmol/L DTT, 0.1% Igepal CA-630, 0.5 mmol/L phenylmethylsulfonyl fluoride]. The minced tissue was homogenized using a Dounce homogenizer and centrifuged at 14,000 rpm at 4°C for 10 minutes. The nuclear pellet obtained was suspended in 0.2 mL buffer B [20 mmol/L HEPES (pH 7.9), 25% glycerol, 420 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 0.5 mmol/L DTT, 0.2 mmol/L EDTA, 4 μmol/L leupeptin, 0.5 mmol/L phenylmethylsulfonyl fluoride] and incubated on ice for 4 hours with intermittent mixing. The suspension was centrifuged at 14,000 rpm at 4°C for 30 minutes. The supernatant (nuclear extract) was collected and stored at −70°C until use. The remaining procedure was followed as described previously for EMSA.

Acknowledgments

We thank Walter Pagel for a careful review of the article.

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