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Honokiol inhibits TNF-α-stimulated NF-κB activation and NF-κB-regulated gene expression through suppression of IKK activation

Anfernee Kai-Wing Tse, Chi-Keung Wan, Xiao-Ling Shen, Mengsu Yang, Wang-Fun Fong *

Bioactive Products Research Group, Department of Biology and Chemistry, City University of Hong Kong, 83 Tat Chee Avenue, Kowloon Tong, Hong Kong SAR, China

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Abstract

Honokiol, a small molecular weight lignan originally isolated from *Magnolia officinalis*, shows anti-angiogenic, anti-invasive and anti-proliferative activities in a variety of cancers. In this study, we investigated whether honokiol affects the transcription factor nuclear factor-kappa B (NF-κB) which controls a large number of genes involved in angiogenesis, metastasis and cell survival. We observed that the tumor necrosis factor-alpha (TNF-α)-induced NF-κB activation was blocked by honokiol in four different cancer cell lines as evidenced by EMSA. Honokiol did not directly affect the NF-κB-DNA binding. Immunoblot experiments demonstrated that honokiol inhibited the TNF-α-stimulated phosphorylation and degradation of the cytosolic NF-κB inhibitor IκBα. Furthermore, honokiol suppressed the intrinsic and TNF-α-stimulated upstream IκB kinases (IKKs) activities measured by a non-radioactive kinase assay using immunoprecipitated IKKs, suggesting a critical role of honokiol in abrogating the phosphorylation and degradation of IκBα. In a HeLa cell NF-κB-dependent luciferase reporter system, honokiol suppressed luciferase expression stimulated by TNF-α and by the transient transfection and expression of NIK (NF-κB-inducing kinase), wild type IKKβ, constitutively active IKKα and IKKβ, or the p65 subunit. Honokiol was also found to inhibit the nuclear translocation and phosphorylation of p65 subunit of NF-κB. RT-PCR results showed that honokiol suppressed NF-κB-regulated inflammatory and carcinogenic gene products including MMP-9, TNF-α, IL-8, ICAM-1 and MCP-1. In line with the observation that NF-κB activation may up-regulate anti-apoptotic genes, it was shown that honokiol enhanced TNF-α-induced apoptotic cell death. In summary, our results demonstrate that honokiol suppresses NF-κB activation and NF-κB-regulated gene expression through the inhibition of IKKs, which provides a possible mechanism for its anti-tumor actions.

Keywords: Honokiol; NF-κB; IκB; IKK; TNF-α; Cell signaling

1. Introduction

Nuclear factor-kappa B (NF-κB) was first described by Sen and Baltimore [1] as a ubiquitous nuclear transcription factor binding to the kappa immunoglobulin-light chain enhancer and subsequently shown to be a regulator in the development of cancer. First, NF-κB mediates tumor promotion, angiogenesis and metastasis through the expression of genes participating in malignant conversion and tumor promotion particularly in inflammation-associated cancer models [2–4]. Second, the activation of genes associated with cell proliferation appears to be imperative for NF-κB-induced oncogenesis. Constitutive activation of NF-κB has been described in a great number of tumors and was found to up-regulate anti-apoptotic genes expression and therefore disrupts the balance between apoptosis and proliferation [2]. Inhibition of NF-κB using the proteasome inhibitor bortezomib leads to anti-multiple myeloma activity and has remarkable anti-tumor activity in preclinical and clinical studies [5]. Third, a variety of cancer therapy

Abbreviations: CHX, cycloheximide; EMSA, electrophoretic mobility shift assay; GST, glutathione *S*-transferase; ICAM-1, intercellular adhesion molecule-1; IL-8, interleukin-8; IκB, inhibitor subunit of NF-κB; IKK, IκB kinase; LPS, lipopolysaccharide; MCP-1, monocyte chemoattractant protein-1; MMP-9, matrix metalloproteinase-9; NF-κB, nuclear factor-kappa B; NIK, NF-κB-inducing kinase; PMA, phorbol myristate acetate; TNF-α, tumor necrosis factor-alpha; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP mick end labeling

* Corresponding author. Tel.: +852 2788 9789; fax: +852 2788 7406.
E-mail address: bhwffong@cityu.edu.hk (W.-F. Fong).

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agents such as TNF-α, taxol and CPT-11 activate NF-κB to result in the expression of genes such as Bcl-2, Bcl-xL, XIAP, c-IAP1 and c-IAP2 that might be responsible for the inhibition of cancer therapy-induced apoptosis [6,7]. In addition to this, the p65 subunit of NF-κB was found to inhibit the function of chemotherapy agents such as 1,25-dihydroxyvitamin D3 by disrupting the binding between transcriptional coactivator (e.g. SRC-1) and the Vitamin D_3 receptor [8,9].

NF-κB encompasses a family of transcription factors includes p65 (RelA), p105/p50, p100/p52, RelB and c-Rel. The classic form of NF-κB is the heterodimer of the p50 and p65 subunits, which contains the transcriptional activation domain and sequestered in the cytoplasm as an inactive complex by the inhibitory proteins IκBs [10]. Chemotherapy agents or acute stimuli such as TNF-α, LPS or PMA lead to the activation of IκB kinases (IKKs) which in turn phosphorylate two key serine residues, Ser^{32} and Ser^{36}, on IκBs within the N-terminal response domain [11]. Phosphorylated IκBs would then undergo ubiquitination and proteolysis by the 26S proteosome, and the release of IκBs unmasks the nuclear localization signal and results in translocation of NF-κB to the nucleus, followed by the activation of specific target genes [11].

Honokiol, a small molecular weight lignan originally isolated from the Chinese medicinal herb *Magnolia officinalis*, has been shown to exhibit many anti-cancer properties. Honokiol has been demonstrated to induce anti-angiogenic activities in human endothelial cells in vitro and was highly effective against angiosarcoma in nude mice [12]. This lignan has also been shown to inhibit tumor invasiveness in vitro, probably by inhibiting matrix metalloproteinase-9 (MMP-9) activity [13]. Honokiol was found to exhibit anti-proliferative and apoptotic activities against a variety of tumor cells including leukemia [14–16], multiple myeloma [17], squamous lung cancer [18], colorectal carcinoma [19,20], and hepatic carcinoma [21,22]. We have previous reported that honokiol enhances the growth inhibition and cell differentiation actions of VD_3 in human leukemia HL-60 cells [23]. In addition to this, honokiol was reported to enhance the cytotoxicity of cancer drugs in B-cell chronic lymphocytic leukemia cells [16] and multiple myeloma cells [17]. These data indicate that honokiol is a potent inducer of apoptosis by itself or in combination with other anticancer agents.

In this study we investigated whether the attenuation of NF-κB activity may account for the anti-cancer effects of honokiol. We observed that honokiol inhibited NF-κB activation through the inhibition of IKK activity, which led to the stabilization of cytoplasmic IκBα and the reduction of nuclear translocation and phosphorylation of the p65 subunit of NF-κB. Honokiol also suppressed NF-κB-dependent reporter gene expression induced by TNF-α and over-expression of transfected NIK, IKK and p65 subunit genes. We also found that honokiol could potentiate the apoptotic activity induced by TNF-α.

2. Materials and methods

2.1. Materials

Honokiol (Fig. 1A), purchased from Wako Pure Chemical Industries, Ltd. (Japan), was dissolved in DMSO as a 100 mM stock solution and stored at −20 °C. LPS (*E. coli* 0127:B8) and PMA were obtained from Sigma (St. Louis, MO, USA). TNF-α from Wako Pure Chemical Industries, Ltd. (Japan) was dissolved in 0.1% (w/v) BSA and stored at −80 °C. [γ-^{32}P] ATP was purchased from Perkin-Elmer Life Sciences (Hong Kong) Ltd. Phospho-p65 (Ser^{536}) and phosphor-IκBα (Ser^{32}) antibodies were purchased from Cell Signaling Technology. NF-κB and Sp1 consensus gel shift oligonucleotides, protein A/G plus-agarose, glutathione *S*-transferase (GST)-IκBα, anti-p65, anti-IκBα, anti-IKKα/β, anti-caspase-3, anti-lamin B, anti-β-tubulin and anti-actin were purchased from Santa Cruz Biotechnology, Inc. AP-1 and OCT-1 consensus gel shift oligonucleotides, Bright-Glo luciferase assay system and Beta-Glo assay system were purchased from Promega.

2.2. Cell culture

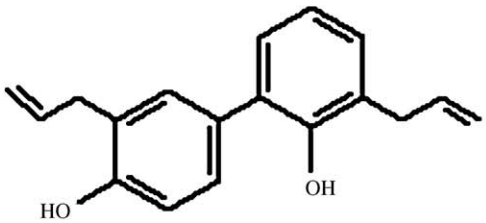
The cell lines used in this experiment were obtained from American Type Culture Collection (Manassas, VA). U937 and HL-60 cells were grown in RPMI-1640 medium containing 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco, NY, USA) at 37 °C in humidified 5% CO_2 atmosphere. MCF-7 and HeLa cells were cultured in Eagles' minimum essential medium containing 10% fetal bovine serum under the same condition.

2.3. Electrophoretic mobility shift assay (EMSA)

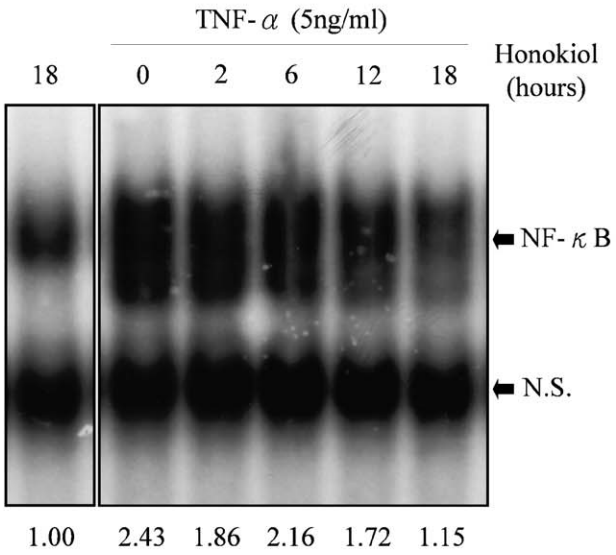
EMSA was performed as described previously [24]. Briefly, equal quantities of nuclear protein (5 μg) from each samples was incubated with radiolabelled gel shift oligonucleotides for 15 min at 37 °C and then resolved on a non-denaturing 5% (w/v) polyacrylamide gel. The gel was dried onto 3 MM blotting paper and used to expose X-ray film for overnight at −70 °C. For supershift assays, 1 μl of antiserum recognizing each of the NF-κB subunits was added to the EMSA reaction 30 min before electrophoresis.

2.4. Western blot analysis

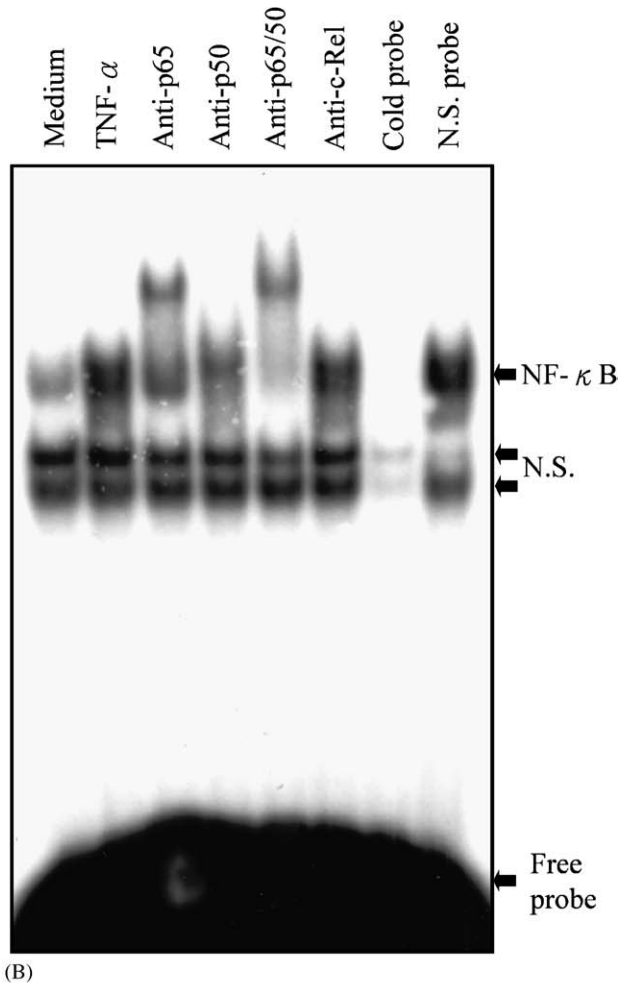
To obtain the whole cell lysates, samples containing 1×10^7 cells were pelleted, washed twice with ice-cold PBS, then lysed in 150 μl of modified RIPA buffer (50 mM Tris–Cl, 1% (v/v) NP-40, 0.35% (w/v) sodium-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, pH 7.4) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM NaF, 1 mM Na_3VO_4, 10 μg/ml each of aprotinin, leupeptin and pepstatin A for 20 min at 4 °C. Supernatants after centrifugation at 14,000 × *g* for 15 min



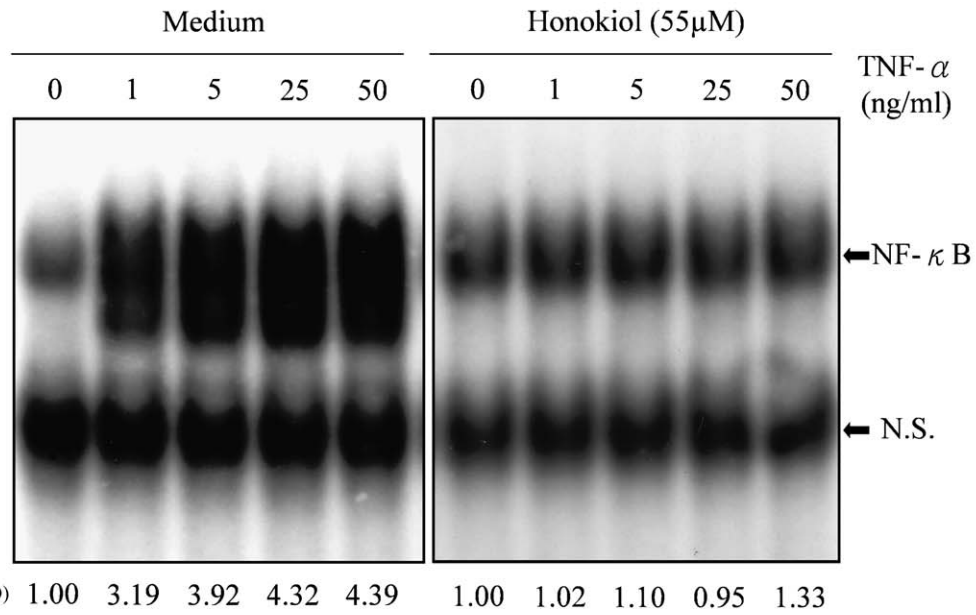
(A) Honokiol



(C)



(B)



(D)

Fig. 1. (A) Structure of honokiol. (B) DNA binding of NF-κB involves p65 and p50 subunits. U937 cells (1.5×10^6/ml) untreated and treated with 5 ng/ml of TNF-α for 30 min and then incubated with indicated antibodies at 37 °C for 30 min. The mixtures were assayed by EMSA. (C) Effects of honokiol pre-treatment on TNF-α-induced NF-κB activities. U937 cells (1.5×10^6/ml) untreated or pre-incubated with 55 μM honokiol for indicated time intervals were treated with 5 ng/ml of TNF-α for 30 min at 37 °C, and then assayed for NF-κB activities by EMSA. (D) Honokiol inhibits NF-κB activities induced by different concentrations of TNF-α. U937 cells (1.5×10^6/ml) untreated or pre-incubated with 55 μM honokiol for 18 h were treated with different concentrations of TNF-α for 30 min at 37 °C, and then assayed for NF-κB activities by EMSA. Densitometry values for the DNA binding (normalized to the corresponding untreated control) are shown *under each lane*. These results are representative of at least three independent experiments.

at 4 °C were collected. Alternatively, cytoplasmic extracts were prepared as previously described [24]. Samples containing 30–50 μg of protein were separated on SDS-polyacrylamide gel and then transferred onto nitrocellulose membrane (0.45 μm, Bio-Rad). Membranes were immunoblotted with primary antibodies and followed by horseradish peroxidase-conjugated secondary antibodies (1:5000) and visualized by ECL (Amersham Biosciences) according to manufacturer's instructions.

2.5. IKK assay

Whole cell lysates (500 μg) were collected in modified RIPA buffer without sodium-deoxycholate, and cellular debris was removed by high-speed centrifugation. Lysates were pre-cleared by incubation with 0.25 μg of the appropriate control IgG together with 20 μl of protein A/G plus (25%, v/v) agarose conjugate for 30 min at 4 °C, followed by centrifugation. Supernatants were then incubated with 1 μg of anti-IKKα/β for 2 h at 4 °C, and then 20 μl of protein A/G plus agarose was added and incubated at 4 °C on a rocker platform overnight. After several washes with IP buffer and PBS, beads containing IKKα/β were incubated with 0.5 μg GST-IκBα substrate, 200 μM ATP in 20 μl kinase buffer (50 mM Tris–Cl pH 7.4, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 1 mM NaF, 1 mM Na_3VO_4, 1 mM PMSF, 0.5 mM DTT, 1 mM benzamidine, 10 μg/ml aprotinin and 1 μg/ml leupeptin) at 30 °C for 30 min. Kinase reactions were stopped by the addition of 5 μl 5× Laemmli's loading buffer and heated at 100 °C for 5 min. The samples were resolved by 8% SDS-PAGE, electro-transferred to nitrocellulose membrane and probed with anti-phosphor-IκBα (Ser^{32}) antibody (1:1000). Membranes were re-probed with anti-IKK to ensure equal loading and presence of total IKK protein.

2.6. Plasmids, transfection and NF-κB-dependent-luciferase reporter assay

The NF-κB-dependent luciferase reporter (p3EnhConA-Luc) and its control vectors (pControl-Luc) were a gift from Dr. Ronald T. Hay (School of Biology, University of St. Andrews, UK). p3EnhConA-Luc is driven by three synthetic copies of the NF-κB-consensus sequence from the immunoglobulin kappa chain promoter. The pControl-Luc is identical to p3EnhConA-Luc excluding the lack of the NF-κB-consensus sequence. β-Galactosidase control vector (pTracer-EF/Bsd/lacZ) was purchased from Invitrogen Life Technologies, Inc. $pCMV_4$-HA-mIκBα encoding both wild type and phosphorylation mutant IκBα and $pCMV_4$-p65 encoding NF-κB subunit p65 [25] were kindly provided by Dr. Warner C. Greene (Gladstone Institute of Virology and Immunology, University of California, USA). The expression plasmids, $pCMV_2$-Flag-IKKβ (WT), $pCMV_2$-Flag-IKKα (SS/EE) and $pCMV_2$-Flag-IKKβ (SS/EE), encoding wild type or constitutively active IKKs were generously supplied by Dr. Richard B. Gaynor (Department of Medicine, University of Texas Southwestern Medical Center, USA) and have been described previously [26]. The pCS3MT-NIK expression plasmid encoding wild type NF-κB-inducing kinase (NIK) was provided by Dr. M. Kracht (Institute of Pharmacology, Medical School Hannover, Germany).

To measure the effect of honokiol on TNF-α-induced NF-κB-dependent gene reporter transcription, HeLa cells were seeded into 24-well plate at a density 1.6×10^5 cells/well for 24 h. Subsequently, cells were transiently transfected with p3EnhConA-Luc or pControl-Luc (0.75 μg) using LipofectAMINE 2000 (Invitrogen). To normalize the transfection efficiency, cells were co-transfected with 0.25 μg of β-galactosidase control vector. After overnight incubation, cells were pre-treated with honokiol for 12 h following by 5 ng/ml TNF-α for 15 h and then harvested with 1× reporter lysis buffer (Promega, Madison, WI). Relative luciferase activity was measured with a Bright-GLO luciferase assay system using POLARStar OPTIMA luminometer (BMG Labtechnologies). Luciferase activity was normalized with β-galactosidase activity, as measured by Beta-GLO luciferase assay system according to the manufacturer's instructions.

To measure the effect of honokiol on NF-κB-dependent gene reporter transcription induced by various kinases, HeLa cells were transfected with p3EnhConA-Luc and β-galactosidase control vector together with 0.2 μg of expression vectors. After 5 h incubation, cells were treated with honokiol for 24 h and then harvested and assayed as described above.

2.7. RT-PCR analysis

RT-PCR was performed to assess NF-κB-regulated gene expression induced by TNF-α, LPS and PMA in U937 cells. One μg of total RNA was subjected to a RT reaction using random oligonucleotide primers and M-MLV reverse transcriptase (Promega). One μl of the RT reaction product was then amplified by PCR using HotStar Taq DNA polymerase (Qiagen) under the following conditions: 95 °C for 1 min; 55 °C for 1 min; and 72 °C for 1 min. The PCR regimen was: 5′-ACCGGAAGGAACCATCT-CACTG-3′, 5′-GCATCTGGCAACCCTACAACA-3′ for IL-8 (444 bp, 25 cycles); 5′-GCTCATAGCAGCCACCTT-CATTC-3′, 5′-TGCAGATTCTTGGGTTGTGGAG-3′ for MCP-1 (297 bp, 25 cycles); 5′-GAGCACTGAAAGCAT-GATCCGGGAC-3′, 5′-TTGGTCTGGTAGGAGACGGC-GATGC-3′ for TNF-α (495 bp, 28 cycles); 5′-TGACCAG-CCCAAGTTGTTGG-3′, 5′-ATCTCTCCTCACCAGCA-CCG-3′ for ICAM-1 (379 bp, 31 cycles); 5′-GGAGACCT-GAGAACCAATCTC-3′, 5′-TCCAATAGGTGATGTTGT-CGT-3′ for MMP-9 (276 bp, 31 cycles); 5′-TGAAGGTC-GGAGTCAACGGATTTGGT-3′, 5′-CATGTGGGCCAT-GAGGTCCACCAC-3′ for GAPDH (983 bp, 28 cycles);.

Five μl of the PCR product were run on a 1% agarose gel and visualized by ethidium bromide staining.

2.8. TUNEL assay

Cleavage of genomic DNA during apoptosis was detected by TUNEL assay using In situ Cell Death Detection Kit (Boehringer Mannheim) and performed according to the manufacturer instruction.

2.9. Morphology assay of live and dead cells

Treated cells were washed twice, resuspended in PBS containing 10 μg/ml acridine orange (AO) and 10 μg/ml ethidium bromide (EB) mixture and visualized immediately under confocal microscope. The status of cells was identified as previous described [27].

2.10. Statistical analysis and data treatment

Statistical analyses were preformed using an unpaired two-tailed Student's *t*-test. Two compounds (A and B) were considered enhancing each other's actions if the effect of combined treatment (AB) was larger then the sum of their individual effects (AB > A + B) after subtraction of the respective background control values.

3. Results

3.1. Inhibited NF-κB binding by honokiol composes of p65 and p50 subunits

NF-κB is a homo- or hetero-dimer composed of different combinations of subunits [28]. The identities of the DNA-retarded protein bands were detected with p65, p50 or c-Rel antibodies and evaluated by EMSA. Both the p65 and the p50 subunits, but not the c-Rel subunit, were shifted to higher molecular weight positions, showing that the activated NF-κB complex consisted of the p65 and p50 subunits (Fig. 1B, lanes 3–5). The identity of the NF-κB complex was confirmed by the disappearance of the band upon competition with 40-fold unlabeled NF-κB consensus probes (Fig. 1B, lane 7). Furthermore, competition with non-specific Sp-1 probes did not alter the NF-κB binding (Fig. 1B, lane 8).

We firstly determined the effect of the length of honokiol pre-treatment time on inhibiting NF-κB activity. U937 cells were incubated with honokiol for various time intervals and subjected to TNF-α treatment. Prolonging honokiol pre-treatment before TNF-α treatment increased the inhibitory effect on NF-κB activation (Fig. 1C).

It has been reported that the activation of NF-κB in leukemic cells by TNF-α is a rapid process and the level of activation depends on the TNF-α doses [29,30]. We investigated the effect of honokiol on NF-κB activation induced by various concentrations of TNF-α. As shown in Fig. 1D, honokiol at 55 μM completely inhibited the NF-κB activation by various doses of TNF-α from 1 to 50 ng/ml.

3.2. Honokiol inhibits TNF-α-induced NF-κB activation in different cell types

To test whether honokiol can block TNF-α-induced NF-κB activation in different cells, Hela (human cervix epithelial), MCF-7 (human breast epithelial), HL-60 (human promyelocytic leukemia) and U937 (human histiocytic lymphoma) cells were pretreated with honokiol for 18 h, stimulated with 5 ng/ml TNF-α for 30 min, isolated the nuclear extracts and the DNA binding of NF-κB was investigated by EMSA. As shown in Fig. 2A–D, honokiol blocked TNF-α-induced NF-κB activation at 55 μM. These results indicate that honokiol inhibits TNF-α-induced NF-κB activation and the effect is not cell type-specific. For most experiments, U-937 cells were used due to the present of both TNF receptors in its cell surface [31] and its well-established methodology in the study of NF-κB pathways.

3.3. Honokiol does not directly modify the NF-κB binding

It has been suggested that many plant-derived chemicals, such as caffeic acid phenethyl ester [32], avicins [33], and andrographolide [34] inhibit NF-κB activity though the directly chemical modification of NF-κB subunits. We incubated nuclear extracts from TNF-α-stimulated cells with increasing concentrations of honokiol at 37 °C for an hour. Our results show that honokiol did not directly alter the NF-κB-DNA binding (Fig. 3A).

We next determined whether honokiol suppresses the activities of other transcription factors, such as AP-1 and OCT-1, by EMSA. As shown in Fig. 3B, honokiol inhibited the binding activity of AP-1, but had minor effect in OCT-1 activity. These results indicate that honokiol would also affect the activities of other transcription factors.

3.4. Honokiol inhibits TNF-α-mediated IκBα phosphorylation and degradation

The kinetics of NF-κB complex in nuclear extracts was evaluated by EMSA. The results shown in Fig. 4A indicate that increases in nuclear NF-κB level were observed after 15–120 min of TNF-α treatment. Treatment with honokiol resulted in diminished nuclear localization of NF-κB complex (Fig. 4A).

TNF-α induces phosphorylation of IκBα which marks it for proteolytic degradation, thereby releasing the active NF-κB dimmers for translocation to the nucleus to activate specific target genes [35]. To elucidate the effect of honokiol on IκBα proteolytic pathway, we examined the phosphorylated and protein levels of IκBα by immunoblot analysis. As shown in Fig. 4B and as previously reported

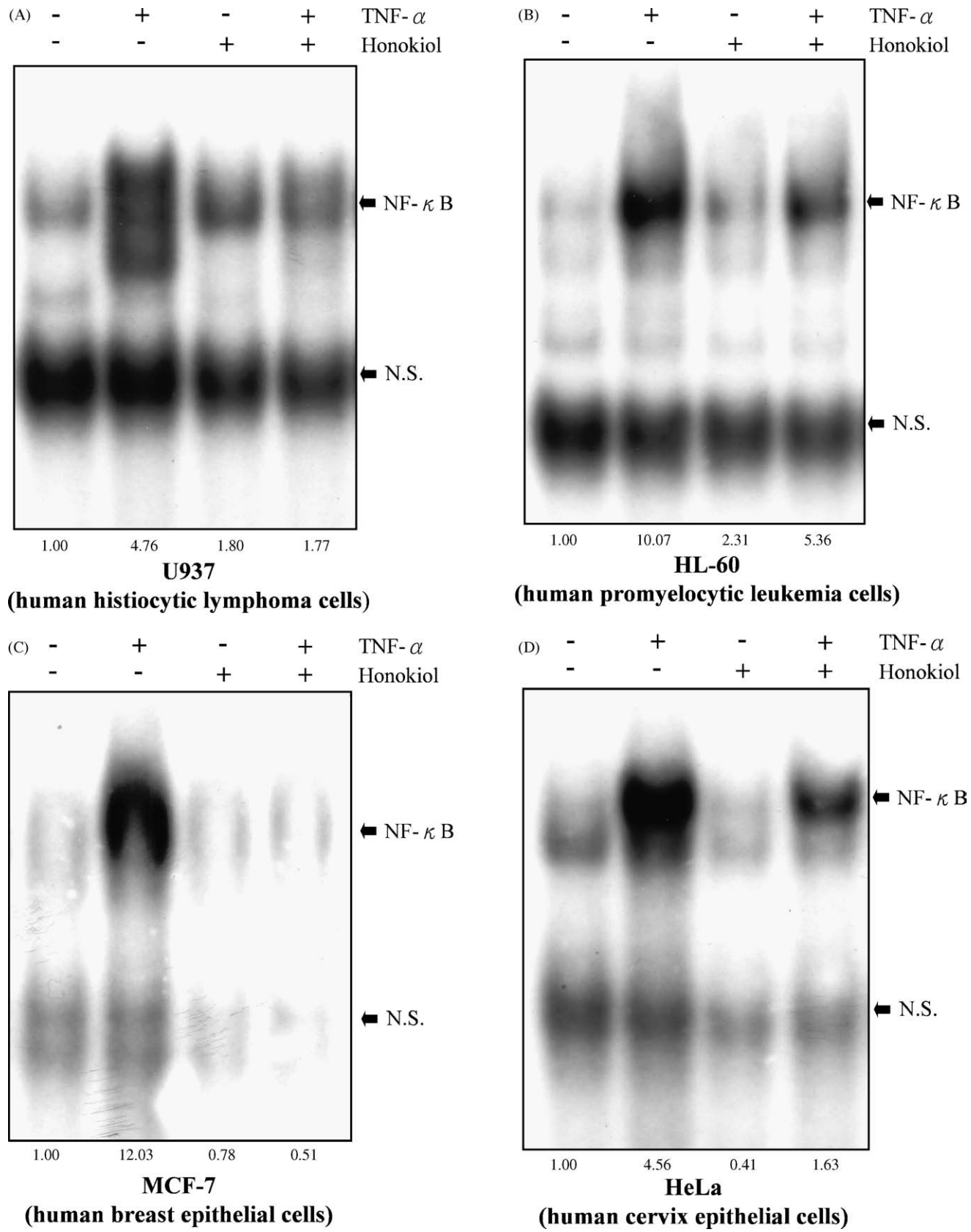


Fig. 2. Honokiol suppresses TNF-α-induced NF-κB activation in different cell lines. U937 (A), HL-60 (B), MCF-7 (C) and HeLa (D) cells were pre-incubated with 55 μM honokiol at 37 °C for 18 h, and followed by stimulation of TNF-α for 30 min. Nuclear extracts were isolated and then analyzed by EMSA described in Section 2. "N.S." represents non-specific binding and densitometry values for the NF-κB binding (normalized to the medium control) are shown *under each lane*. These results are representative of at least three independent experiments.

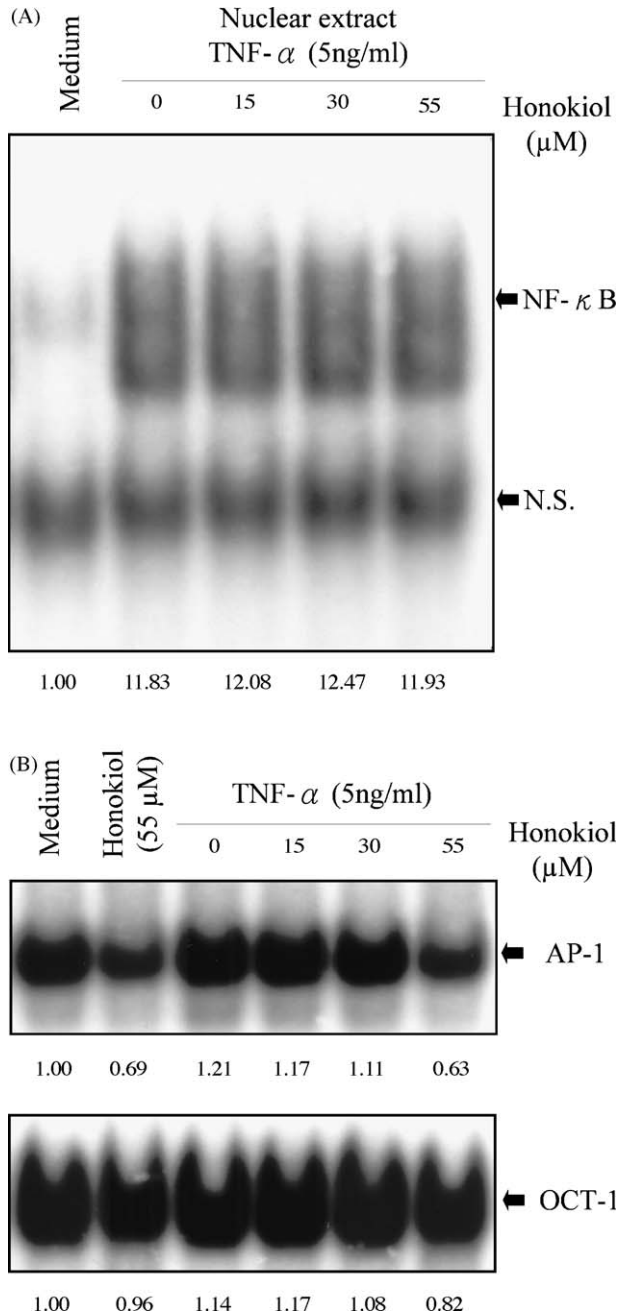


Fig. 3. (A) Honokiol does not directly modify the NF-κB binding. Nuclear extracts from TNF-α treated U937 cells were incubated with indicated concentrations of honokiol for 1 h at 37 °C, and then assayed for NF-κB binding by EMSA. (B) Effects of honokiol on AP-1 and OCT-1 activities. U937 cells (1.5×10^6/ml) untreated or pre-incubated with different concentrations of honokiol for 18 h were incubated with 5 ng/ml of TNF-α for 30 min at 37 °C and then assayed for AP-1 and OCT-1 binding activities by EMSA. These results are representative of at least three independent experiments.

[36], TNF-α caused a rapid degradation of IκBα after a 5 min treatment, which was followed by a slow but dramatic restoration of the IκBα level at 120 min. Honokiol at 55 μM completely blocked the degradation of IκBα (Fig. 4B) To evaluate the level of IκBα phosphorylation we used proteasomal inhibitor MG132 to block degradation of phosphorylated IκBα [37], and the phosphorylation status was assessed by immunoblot with an IκBα phospho-Ser^{32} specific antibody. At 30 min of treatment, honokiol inhibited the TNF-α-induced phosphorylation of IκBα (Fig. 4C). These results demonstrate that honokiol is effective in the blockage of IκBα proteolytic pathway in NF-κB activation.

3.5. Honokiol inhibits TNF-α-induced IKK activity

Previous studies suggested that TNF-α induces IκBα phosphorylation and degradation via activation of the IKK complex [38]. We performed a non-radioactive in vitro kinase assay using anti-IKK-antibody-precipitated protein from cell lysates as an enzyme source and GST-IκBα as the substrate. TNF-α treatment stimulated IKK kinase activity and the maximum phosphorylation activity was achieved in about 5 min of treatment (Fig. 5A). Honokiol inhibited both the intrinsic and TNF-α-induced IKK activity in U937 cells (Fig. 5B) but did not affect the immuno-precipitated IKK protein levels (Fig. 5B, lower panel). Direct cell-free IKK kinase assay with lysates obtained from TNF-α-treated cells showed that at 55 μM honokiol had only a minor direct effect on IKK activity (Fig. 5C).

We then investigated the capacity of honokiol to inhibit NF-κB-dependent reporter gene expression induced by over-expression of the two catalytic IKK subunits IKKα and IKKβ and also NF-κB-inducing kinase (NIK), which is a major component in the TNF-receptor-induced IKK activation pathway [39]. NF-κB-dependent luciferase activity was increased with the transient transfection of NIK, wild-type IKKβ, constitutively active IKKα and IKKβ and p65 plasmids into HeLa cells (Fig. 5D). Honokiol significantly inhibited the NF-κB-dependent gene expression in all of the transfected cells (Fig. 5D). These results indicate that honokiol inhibit IKK-mediated NF-κB activation pathway by inhibiting both the intrinsically expressed and the extrinsically stimulated IKK activity.

3.6. Honokiol inhibits TNF-α-induced p65 nuclear accumulation and phosphorylation

Since p65 subunit is responsible for the transcriptional activity of NF-κB [40], we determined the p65 nuclear accumulation using Western blot analysis. As illustrated in Fig. 6A, honokiol was able to inhibit the levels of p65 in the nucleus with TNF-α treatment up to 120 min, and the inhibitory effects were dose dependent (Fig. 6B). These results are in line with observations mentioned above that honokiol interferes with TNF-α-mediated degradation of IκBα and the sequestration of NF-κB complex in cytoplasm (Fig. 4).

It has been known that phosphorylation of the p65 subunit by a variety of kinases leads to the modification in NF-κB transcriptional activity [41]. Results showed that honokiol completely blocked TNF-α-mediated p65 phosphorylation in whole cell extracts (Fig. 6C).

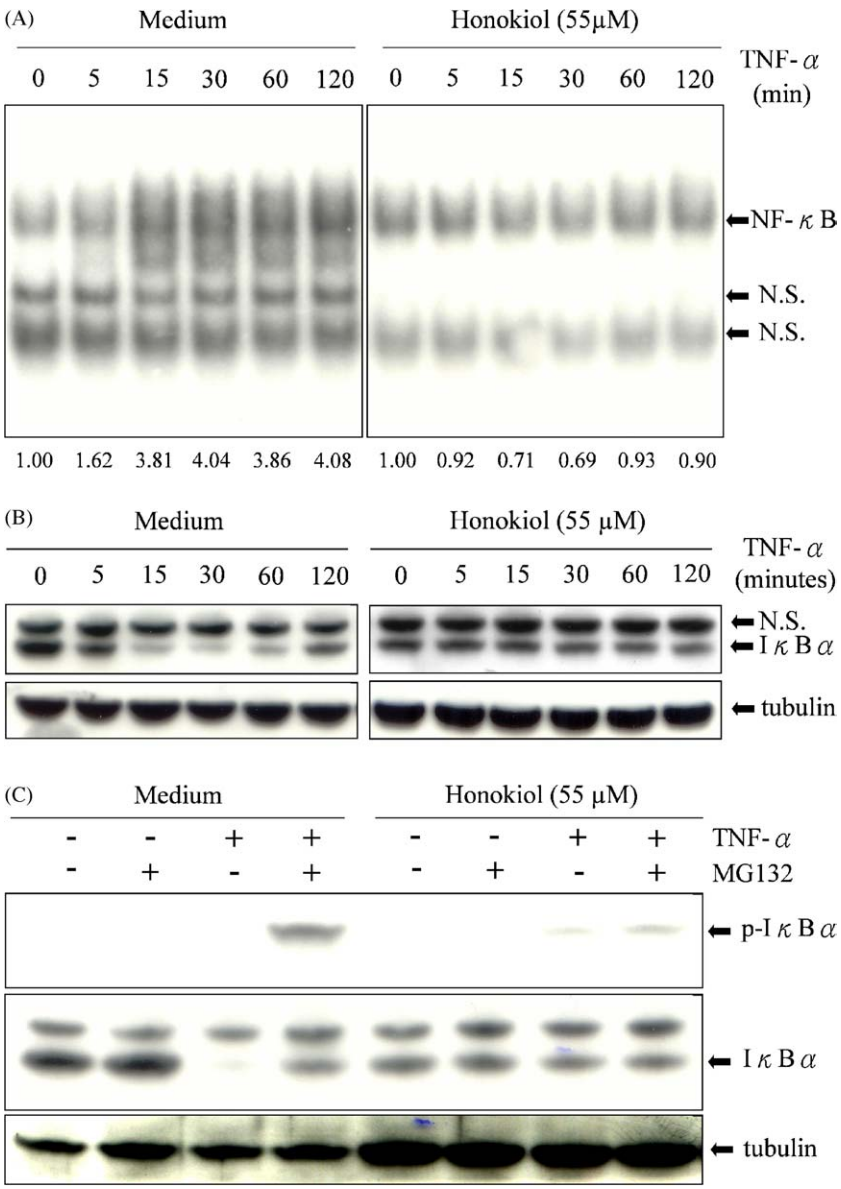


Fig. 4. Honokiol inhibits TNF-α-induced degradation and phosphorylation of IκBα. (A) Honokiol blocks TNF-α-induced time-dependent NF-κB activation. U937 cells (1.5×10^6/ml) were untreated or treated with 55 μM honokiol for 18 h, and then stimulated with 5 ng/ml of TNF-α for indicated time intervals at 37 °C. The NF-κB activities were assayed by EMSA. Densitometry values for the NF-κB binding (normalized to the corresponding untreated control) are shown *under each lane*. (B) Honokiol inhibits TNF-α-induced degradation of IκBα. U937 cells (1.5×10^6/ml) were untreated or treated with 55 μM honokiol for 18 h and then stimulated with 5 ng/ml of TNF-α at indicated time intervals. The cytosolic extracts were isolated and subjected to Western blot analysis with anti-IκBα antibody, as described under Section 2. (C) Honokiol blocked the TNF-α-stimulated phosphorylation of IκBα. U937 cells (1.5×10^6/ml) were untreated or treated with 55 μM honokiol for 18 h, followed by 1 h treatment with 20 μM protease inhibitor MG132, and then stimulated with 5 ng/ml of TNF-α for 30 min. Whole cell extracts were prepared, fractionated and assayed by Western blot analysis using anti-p-IκBα antibody. These results are representative of at least three independent experiments.

3.7. Honokiol inhibited NF-κB-dependent luciferase gene expression induced by TNF-α

To detect the effect of honokiol on TNF-α-induced NF-κB dependent gene expression, HeLa cells were transiently transfected with a NF-κB promoter-dependent luciferase reporter construct and stimulated with 5 ng/ml TNF-α. Treatment with TNF-α resulted in a 4.3-fold increase in reporter gene expression, which was repressed by pre-treatment of honokiol to 0.6-fold with comparing to control (Fig. 7). Co-transfection of IκBα plasmid abrogated the luciferase gene expression induced by TNF-α (Fig. 7, lane 5). The specificity of NF-kB-dependent gene expression was addressed by lack of response in cells transfected with promoter-less control reporter (Fig. 7, lane 6).

3.8. Honokiol represses NF-κB-regulated gene expression induced by TNF-α, LPS and PMA

Activation of NF-κB by various stimuli, such as TNF-α, LPS or PMA, induces the expression of diverse target genes that regulate tumorigenesis [42]. Whether pre-treat-

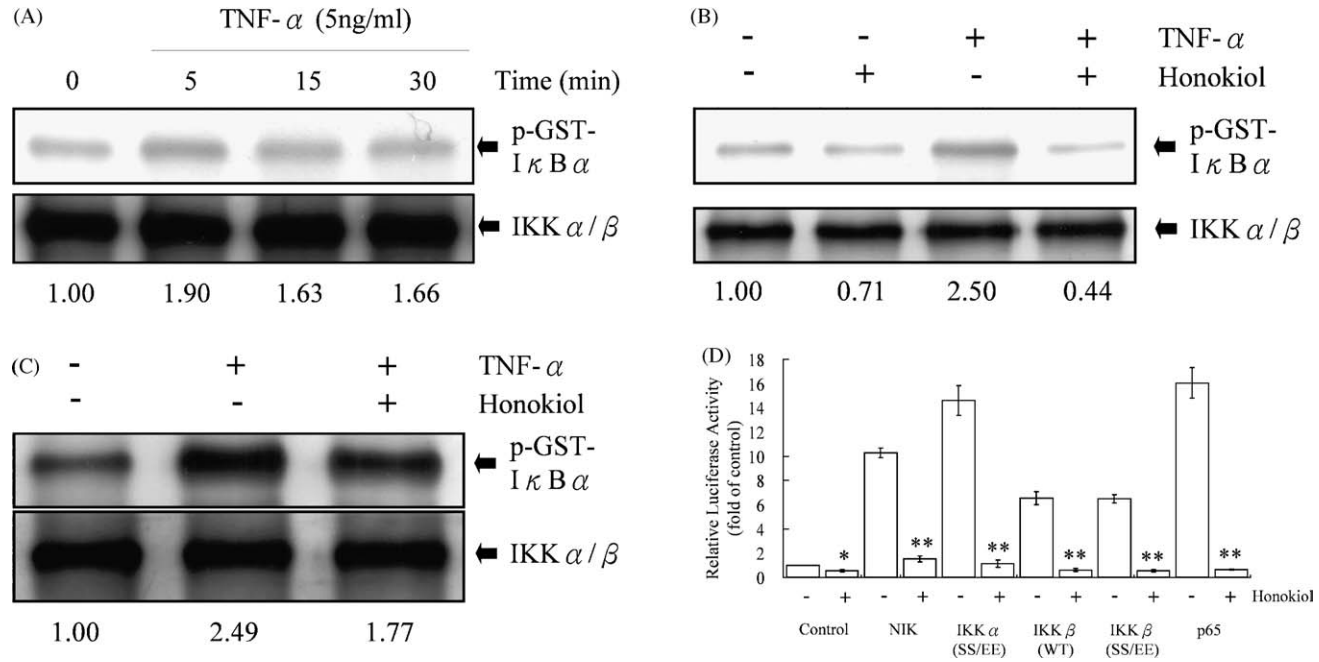


Fig. 5. Effect of honokiol on the TNF-α-induced IKK activation. (A) TNF-α-induced time-dependent IκBα phosphorylation. Whole cell lysates of U937 cells (1.5×10^6/ml) untreated or treated with 5 ng/ml of TNF-α for different time periods were immunoprecipitated using anti-IKKα/β antibody, and subsequently subjected to IKK kinase assay. To examine the phosphorylation of GST-IκBα, reaction complex was assayed by Western blot analysis with p-IκBα antibody. The same nitrocellulose membrane was re-probed with anti-IKKα/β antibody to detect the level of immunoprecipitated IKK protein. (B) Honokiol inhibits TNF-α-induced IKK activity. U937 cells (1.5×10^6/ml) were untreated or treated with 55 μM honokiol for 18 h, then stimulated with 5 ng/ml of TNF-α for 5 min, IKK protein were immunoprecipitated with anti-IKKα/β antibody. The level of phosphorylation of GST-IκBα was detected by IKK assay. (C) Direct effect of honokiol on TNF-α-stimulated IKK activity. U937 cells (1.5×10^6/ml) were untreated or treated with 5 ng/ml of TNF-α for 5 min, and the whole cell extracts were prepared and immunoprecipitated with anti-IKKα/β antibody. The IKK assay was performed in the absence or presence of 55 μM honokiol for 30 min at 30 °C. Densitometry values for the IKK activities (normalized to IKKα/β protein and the medium control) are shown *under each lane*. These results are representative of at least three independent experiments. (D) Honokiol inhibits NF-κB-dependent gene expression induced by over-expression of NIK, IKKα/β and p65 subunit. HeLa cells were transient transfect with NF-κB-dependent gene reporter along with the 200 ng of NIK, wild-type IKKβ, constitutively active IKKα/β and p65 plasmids, and then incubated with 55 μM honokiol for 24 h. The luciferase activities were normalized with β-galactosidase activity and the results are expressed as fold activity to medium control. Results are mean ± standard derivation of three independent experiments. Statistically significant differences between with or without honokiol treatments: $^{*}p < 0.002$, $^{**}p < 0.0001$.

ment of honokiol represses the NF-κB-regulated gene expression by various stimuli was examined by RT-PCR. Treatment of cells with honokiol suppressed either the intrinsic or induced expression of NF-κB-regulated gene products (Fig. 8).

3.9. Honokiol sensitizes TNF-α-induced apoptosis

TNF-α has dual actions on apoptosis. On the one hand it triggers the death receptor-mediated pro-apoptotic pathways, but on the other it may up-regulate NF-κB-regulated anti-apoptotic genes, which results in the blockage of cell death [7]. Thus, the sensitivity of cells to apoptotic signals can be increased by the inhibition of NF-κB-mediated survival and anti-apoptotic pathways. To investigate whether honokiol can sensitize TNF-α-induced cell death via apoptosis, we first examined the DNA fragmentation by TUNEL assay coupled with flow cytometry. As demonstrated in Fig. 9A, honokiol enhanced the apoptotic effects by TNF-α. The enhancing apoptotic effect was also confirmed by the caspase-3 cleavage (Fig. 9B) and the live/dead morphological assay (Fig. 9C). All the experiments confirmed that honokiol enhanced the apoptotic effect of TNF-α.

4. Discussion

The bark of *M. officinalis* is a rich source for honokiol [43] and has been wildly used as a folk remedy for gastrointestinal disorders, cough, anxiety, allergy and other diseases in China, Korea and Japan. In the present study, we demonstrated that honokiol suppressed the TNF-α-stimulated DNA binding activities of NF-κB in four different cell types. Mechanistically, we showed that honokiol inhibited intrinsically over-expressed or extrinsically stimulated IKK activity, which in turn affected IκBα phosphorylation and degradation, and p65 phosphorylation and translocation. Moreover, the suppression of NF-κB activation by honokiol also resulted in the down-regulation of various NF-κB-regulated gene products induced by different stimuli and the enhancement of TNF-α-induced apoptosis.

Our data suggest that honokiol affects IKK or the signal pathways and kinases upstream of IKK. Honokiol has an

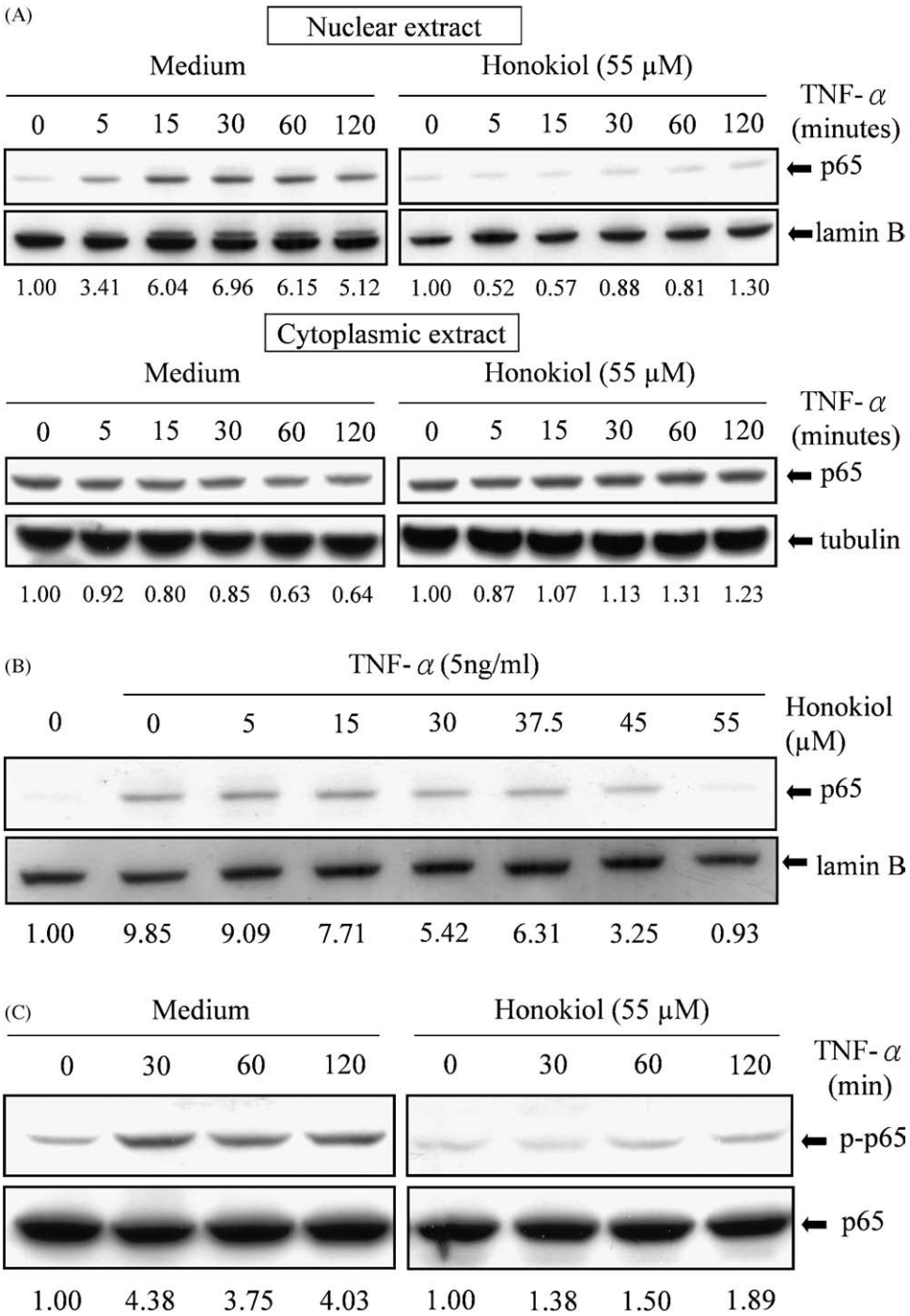


Fig. 6. Honokiol inhibits p65 nuclear translocation and phosphorylation induced by TNF-α. (A) Honokiol inhibits TNF-α-induced p65 nuclear translocation. U937 cells (1.5×10^6/ml) were untreated or treated with 55 μM honokiol for 18 h, and then stimulated with 5 ng/ml of TNF-α for indicated time intervals at 37 °C. Nuclear and cytoplasmic extracts were prepared and the p65 protein levels were measured by Western blot analysis as described under Section 2. Densitometry values for the p65 protein levels (normalized to lamin B or tubulin and the corresponding untreated control) are shown *under each lane*. (B) Dose-dependent inhibition of p65 nuclear translocation by honokiol. U937 cells (1.5×10^6/ml) were untreated or treated with indicated concentrations of honokiol for 18 h before stimulated with 5 ng/ml of TNF-α for 30 min at 37 °C. Nuclear extracts were prepared and the p65 protein levels were measured by Western blot analysis. Densitometry values for the p65 protein levels (normalized to lamin B and the medium control) are shown *under each lane*. (C) Honokiol inhibits p65 phosphorylation induced by TNF-α. U937 cells (1.5×10^6/ml) were untreated or treated with 55 μM honokiol for 18 h, and followed by stimulation with 5 ng/ml of TNF-α for various time periods. Whole cell extracts were prepared, fractionated and assayed by Western blot analysis using anti-p-p65 antibody. Densitometry values for the p-p65 protein levels (normalized to total p65 and the corresponding untreated control) are shown *under each lane*. These results are representative of at least three independent experiments.

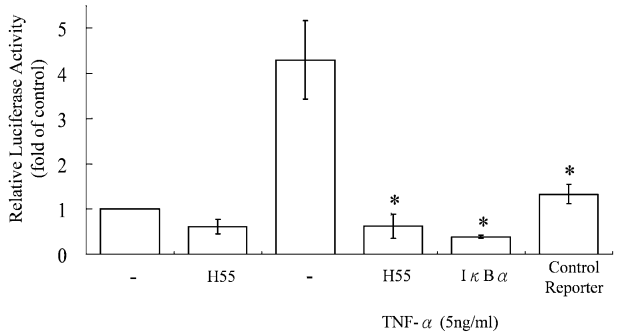


Fig. 7. Honokiol inhibits NF-κB-dependent luciferase reporter gene expression induced by TNF-α. HeLa cells were transient transfect with NF-κB-dependent gene reporter (p3EnhConA-Luc), followed by untreated or 12 h 55 μM honokiol (H55) pretreatment and then 5 ng/ml TNF-α stimulation for 15 h. The luciferase activities were normalized with β-galactosidase activity and the results are expressed as fold activity to medium control. Results are mean ± standard derivation of three independent experiments. Statistically significant differences between TNF-α stimulation alone and other treatments: $^{*}p < 0.005$.

effect on the intrinsically expressed IKK and in addition it also inhibits NIK-induced NF-κB activation. The IKK complex, consisting of two catalytic subunits IKKα and IKKβ and a regulatory subunit IKKγ, targets at two distinct

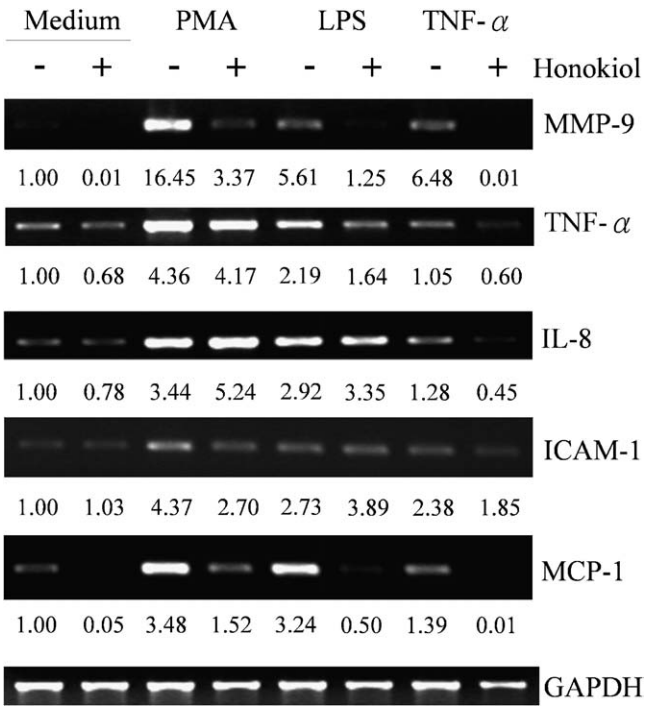


Fig. 8. Honokiol repressed NF-κB-regulated gene expression induced by TNF-α, LPS and PMA. U937 cells (1.0×10^6/ml) with or without honokiol pretreatment (55 μM, 12 h) were stimulated with PMA (25 ng/ml), LPS (1 μg/ml) or TNF-α (5 ng/ml) for 12 h. The mRNA level of NF-κB-regulated genes matrix metalloproteinase-9 (MMP-9), tumor necrosis factor-α (TNF-α)m interleukin-8 (IL-8), intercellular adhesion molecule-1 (ICAM-1), and monocyte chemotactic protein-1 (MCP-1) were examined using RT-PCR assay as described under Section 2. Densitometry values for various gene expressions (normalized to GAPDH levels and the medium control) are shown *under each lane*. These results are representative of at least three independent experiments.

serine residues on IκBα. The phosphorylation of the serine residues leads to a rapid ubiquitin-dependent proteolysis of IκBα and finally NF-κB activation [28,38]. In a HeLa cell NF-κB-regulated luciferase reporter system, we have demonstrated that honokiol suppresses the reporter expression stimulated by the transfection and expression of the wild-type IKKβ, and constitutively active IKKα and IKKβ. Phosphorylation/activation of IKK involves multiple upstream kinases including NIK, MEKK3, Tpl2, PKC, and Akt [28] and the exact molecular targets affected by honokiol remain to be identified. Inhibition of constitutively active IKK leads to the promotion of apoptosis in many types of cancer such as leukemia [44], lung cancer [45] and malignant melanoma [46]. We have demonstrated that honokiol inhibited both constitutive and stimulus-induced IKK activities, which may account for the anti-tumor effects of honokiol on various cancer cell lines.

Consistent with previous reports [47–49], honokiol suppresses inflammatory gene products IL-8, MCP-1, TNF-α, ICAM-1, and MMP-9 that are known to be regulated by NF-κB. The inhibited inflammatory genes in this study have been found to participate in tumorigenesis processes. IL-8 and MCP-1 are shown to be up-regulated in ovarian carcinoma and play important roles in angiogenesis, invasion, autocrine growth loops and resistance to apoptosis [50]. ICAM-1 and MMP-9 are essential in tumor cell invasion process, given the evidences that protections of metastasis were observed in ICAM-1 mutant mice [51] or MMP-9-deficient mice [52].

Specific inhibition of NF-κB-regulated inflammatory responses by honokiol was observed in RT-PCR gene expression experiment. For instance, honokiol reduces ICAM-1 mRNA levels in PMA-stimulated cells, but not in cells stimulated with LPS (Fig. 8). However, as suggested by Baltimore and co-workers [53–55], the stimulus responsiveness of NF-κB is not solely dependent on the DNA-NF-κB interactions but in addition the interaction duration, or the participation of other transcriptional machinery, such as neighboring transcription factors, co-activators and chromatin components, may make various contributions to the formation of the final responses. Thus, honokiol may influence individual stimulus responsiveness by affecting distinct regulatory factors in NF-κB activation. In addition to this, ICAM-1 promoter can be also activated by other transcription factors include AP-1, CCAAT/enhancer-binding protein, Ets, signal transducer and activator of transcription (STAT) or SP1 [56]. Given that honokiol also influences other transcription factors-regulated signaling pathways (Fig. 3B), which may explain its individual inhibitory effects on different stimuli. Additionally, honokiol appeared to be more consistently in the disruption of TNF-α-stimulated gene expressions than LPS or PMA (Fig. 8). Hayakawa et al. has reported that *N*-acetyl-L-cysteine selectively blocks TNF-α signaling by reducing the affinity of receptor to TNF-α independently of anti-oxidative function [57]. In our study, the role of

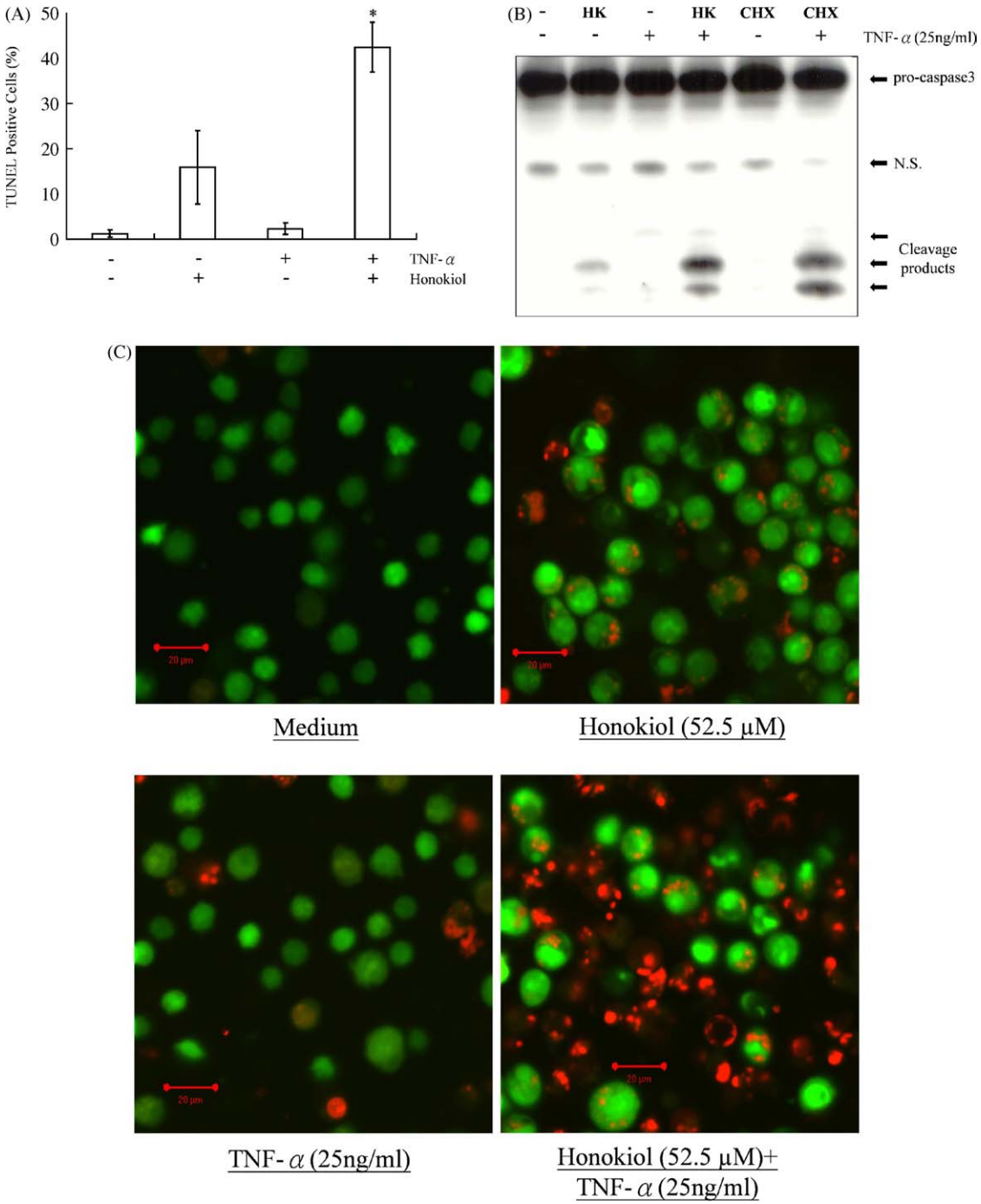


Fig. 9. Honokiol sensitizes TNF-α-induced apoptosis in U937 cells. (A) Honokiol enhances TNF-α-induced TUNEL positive cells. U937 cells (1.0×10^6/ml) pretreated with 52.5 μM honokiol for 4 h and then incubated with 25 ng/ml TNF-α for 24 h. The TUNEL positive cells were identified by flow cytometry as described under Section 2. Results are mean ± standard derivation of three independent experiments. Statistically significant differences from the sum of the individual effects of drugs: $^{*}p < 0.01$. (B) Augmentation of TNF-α-induced capases-3 activation by honokiol. U937 cells (1.0×10^6/ml) were treated as described in (A) and the pro- and cleaved caspase-3 proteins were detected by Western blot analysis using anti-caspase-3 antibody. Cycloheximide (CHX, 1 μg/ml) was employed as the positive control for the caspase-3 cleavage. These results are representative of at least three independent experiments. (C) Effect of honokiol on TNF-α-induced apoptotic nuclear condensation. U937 cells (1.0×10^6/ml) were treated as described in (A). Cells were stained with AO/EB and analyzed by confocal laser microscopy as described under Section 2.

honokiol-induced IKK inactivation in the blockage of NF-κB activation was verified by the inhibition of intrinsic IKK activity (Fig. 5B) and extrinsic IKK-regulated NF-κB activation (Fig. 5D). It is important to note, however, honokiol has been identified as a modulator of the GABAA receptors in vitro [58,59], it is possible that the inhibition of TNF-α-induced NF-κB activation by honokiol is partially through the influence on the TNF receptor binding events.

On the cellular level we have shown that one of honokiol notable actions is the enhancement of TNF-α-induced

apoptosis (Fig. 9). A number of mechanisms have been suggested in the enhancement of TNF-α-induced cell death: (i) suppression of NF-κB-regulated anti-apoptotic signaling [60–62]; (ii) activation of caspase-8, the triggering caspase in the TNF-α apoptotic pathway [63]; (iii) promotion of c-Jun N-terminal kinase signaling (JNK) [64]; (iv) accumulation of reactive oxygen species [65,66]; (v) inhibition of p38 mitogen-activated protein kinase activation [67,68]; (vi) inhibition of protein synthesis [69], specifically the expression of NF-κB-regulated apoptotic proteins [70]. Honokiol has been reported to activate caspase-8 [16,17], inhibit the protein synthesis [71] and down-regulate anti-apoptotic gene expression [18,20]. The involvement of other pathways in the enhancement of TNF-α-induced apoptosis by honokiol needs to be further clarified.

Honokiol is likely a universal inhibitor for NF-κB activation in various cell-types. In recent studies, cell type-specific inhibition of TNF-α-induced NF-κB activation was observed, probably through specifically regulation NF-κB signaling mediators such as Akt [72] or reactive oxygen species [73] in stimulated cells. Honokiol in this study blocks TNF-α-induced NF-κB activation in all representative cell lines with a lack of cell type specificity. Therefore, honokiol is a potent universal inhibitor for NF-κB activation.

We found that honokiol also inhibits AP-1 binding activity as shown in EMSA (Fig. 3A). Similar effects were observed in other plant-derived lignans such as dihydroguaiaretic acid [74], sauchinone [75], and arctigenin [76]. These findings suggest that honokiol and other lignans may also elicit potential pharmacological actions on other transcriptional factor-regulated signal transduction pathways. However, the precise mechanisms of inhibitory actions on AP-1 and other transcription factors remain to be elucidated.

Intraperitoneal administration of a single dose of 250 mg/kg of honokiol yielded maximum plasma concentration of >3.5 mM with an elimination half-life of 5.2 h [19]. After multiple doses of 2–3 mg/mice at 1–2 days intervals, honokiol was effective in the inhibition of tumor growth in vivo [12,19]. Thus, concentrations of honokiol used in our studies are comparable and achievable with that used in animal's studies.

In summary, these experiments demonstrate that honokiol inhibits TNF-α-induced NF-κB activation via inhibition of IKK activation, which leads to the abrogation of phosphorylation and degradation of IκBα, translocation and phosphorylation of p65 subunit and NF-κB-dependent reporter gene expression. These effects possibly will correspond to the regulation of NF-κB-regulated inflammatory and carcinogenic gene expression and augmentation of TNF-α-induced apoptosis. This study extends our understanding on the molecular mechanisms underlying the anti-tumor activities of honokiol. Given that honokiol is effective in the inhibition of tumor growth in vivo [12,19], this lignan could serve as potent compound in the treatment of cancer.

Acknowledgements

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