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# Inhibitory Effect of Magnolol on TPA-Induced Skin Inflammation and Tumor Promotion in Mice

Daih-Huang Kuo,*,† You-Syuan Lai,‡ Chih-Yu Lo,§ An-Chin Cheng,∥ Hou Wu,⊥ and Min-Hsiung Pan*,‡

†Department of Pharmacy and Graduate Institute of Pharmaceutical Technology, Tajen University, Pingtung, Taiwan, ‡Department of Seafood Science, National Kaohsiung Marine University, Kaohsiung, Taiwan, §Department of Food Science, National Chiayi University, Chiayi City 60004, Taiwan, ∥Department of Nutrition and Health Sciences, Chang Jung Christian University, Tainan 71101, Taiwan, and ⊥Department of Food Science, Rutgers University, New Brunswick, New Jersey 08901-8520

Magnolol has been reported to have an anti-inflammatory and antitumor effect in vitro and in vivo. Herein, we report the investigation of the inhibitory effects of magnolol on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in mouse skin. We found that the topical application of magnolol effectively inhibited the transcriptional activation of iNOS and COX-2 mRNA and proteins in mouse skin stimulated by TPA. Pretreatment with magnolol resulted in the reduction of TPA-induced nuclear translocation of the nuclear factor-$\kappa$B (NF$\kappa$B) subunit and DNA binding by blocking the phosphorylation of I$\kappa$B$\alpha$ and p65 and subsequent degradation of I$\kappa$B$\alpha$. In addition, magnolol can suppress TPA-induced activation of extracellular signal-regulated kinase (ERK)1/2, p38 mitogen-activated protein kinase (MAPK), and phosphatidylinositol 3-kinase (PI3K)/Akt, which are upstream of NF$\kappa$B. Moreover, magnolol significantly inhibited 7,12-dimethylbene[*a*]anthracene (DMBA)/TPA-induced skin tumor formation by reducing the tumor multiplicity, tumor incidence, and tumor size of papillomas at 20 weeks. All these results revealed that magnolol is an effective antitumor agent and that its inhibitory effect is through the down-regulation of inflammatory iNOS and COX-2 gene expression in mouse skin, suggesting that magnolol is a novel functional agent capable of preventing inflammation-associated tumorigenesis.

**KEYWORDS: Magnolol; TPA; skin inflammation; tumorigenesis**

## INTRODUCTION

Magnolol (**Figure 1**) is the main constituent of the stem bark of *Magnolia obovata*, widely used as a folk remedy for gastrointestinal disorders, cough, diarrhea, anxiety, and allergic diseases (*1*). Several recent studies reported that magnolol has a number of diverse pharmacological effects including inducing apoptosis (*2*), autophagy (*3*), antioxidative (*4*), anticarcinogenic, and antitumor activities (*5*). Furthermore, magnolol has a board spectrum anti-inflammatory effect (*6*, *7*). It was demonstrated to be ablt to suppress the expression of the inducible nitric oxide synthase (iNOS) in macrophages (*8*) and inhibit the formation of eicosanoids probably through inhibition of phospholipase A2, cyclooxyganase, and 5-lipoxygenase (*9*).

It has been known that inflammation is causally linked to carcinogenesis and acts as a driving force in premalignant and malignant transformation of cells (*10*). Topical application of TPA to DMBA-initiated mice leads to edema and papilloma formation by enhancing iNOS and COX-2 protein expression. Specific iNOS and COX-2 inhibitors are able to counteract the biological events (*11*). Activated NF$\kappa$B often facilitates the transcription of numerous genes, including iNOS and COX-2, resulting in inflammation and tumorigenesis. Activation of NF$\kappa$B by TPA is induced by a cascade of events leading to the activation of inhibitor $\kappa$B (I$\kappa$B) kinases (IKKs), which in turn phosphorylates I$\kappa$B. The subsequent ubiquitination and proteasomal degradation of I$\kappa$B lead NF$\kappa$B free to translocate to the nucleus (*12*). These kinases can be activated through phosphorylation by upstream kinases, including NF$\kappa$B-inducing kinase, mitogen-activated protein kinase, and protein kinase C (*13*). In addition, many studies have confirmed the cytokine function in the induction of the transcription activity of NF$\kappa$B through ERK1/2 (p42/44), p38 MAPK, and PI3K/Akt pathways (*14*, *15*). More importantly, iNOS has been shown to be involved in regulating cycoloxygenase-2 (COX-2), which plays a pivotal role in colon tumorigenesis (*16*). These observations suggest that iNOS may exacerbate turmorigenesis.

*To whom correspondence should be addressed. (D.-H.K.) No.20, Weisin Rd., Sin-er Village, Yanpu Township, Pingtung County 907, Taiwan. E-mail: kuo0939@gmail.com. (M.-H.P.) Department of Seafood Science, National Kaohsiung Marine University, No.142, Haijhuan Rd., Nanzih District, Kaohsiung 81143, Taiwan. Tel: (886)-7-361-7141 ext. 3623. Fax: (886)-7-361-1261. E-mail: mhpan@mail.nkmu.edu.tw.



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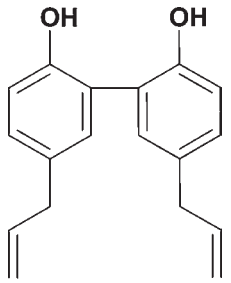


**Figure 1.** Chemical structure of magnolol.

We investigated whether the attenuation of NF$\kappa$B activity may account for the anti-inflammatory and antitumor promotion effects of magnolol. In a previous study, magnolol was shown to reduce the nuclear NF$\kappa$B content in TNF-$\alpha$-stimulated human U937 promonocytes cells (*17*). However, the exact molecular mechanisms underlying the chemopreventive effect of magnolol remain largely unresolved. In this research, we have studied the effect of magnolol on the TPA-induced iNOS and COX-2 expression in mouse skin, explored underlying molecular mechanisms, tested the anti-inflammatory activity of magnolol in mouse skin following TPA application, and investigated the inhibitory effect of magnolol on mouse skin tumor promotion using a two-stage carcinogenesis model including tumor incidence, multiplicity, and volume.

## MATERIALS AND METHODS

**Chemicals and Animals.** Magnolol (purity >98%), TPA, and dimethylbenz(*a*)anthracene (DMBA) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used were in the purest form available commercially. Female ICR mice at 5−6 weeks old were supplied from the BioLASCO Experimental Animal Center (Taiwan Co., Ltd.). All animals were housed in a controlled atmosphere (25 ± 1 °C at 50% relative humidity) and with a 12-h light/12-h dark cycle. The dorsal skin of each mouse was shaved with surgical clippers before the application of tested compound. Magnolol and TPA were dissolved in 200 $\mu$L of acetone and applied topically to the shaved area of each mouse.

**Western Blot Analysis.** The female ICR mice were topically treated on their shaved backs with magnolol in 200 $\mu$L of acetone and 30 min prior to 10 nmol TPA treatment. The mice were scarified by cervical dislocation at the indicated times. Protein from mouse skin was isolated according to our previous work(*18*) . Protein concentrations were measured by the Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). The Western blot assay was performed according our previous work (*18*). Briefly, equal amounts of protein (30−50 $\mu$g) were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and transferred onto the 45 $\mu$m immobile membrane (polyvinylidene difluoride; Millipore Corp., Bedford, MA). The membranes were blocked with blocking solution. The membrane was further incubated with the respective specific antibodies, at appropriate dilution (1: 1000) using blocking solution with the primary antibody of iNOS, I$\kappa$B$\alpha$, p50, p65, and phospho-PI3K(Tyr508) polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), COX-2 monoclonal antibodies (Transduction Laboratories, BD), phospho-p65 (Ser536), phospho-p38 (Thr180/Tyr182), phospho-ERK1/2 (Thr202/Tyr204), ERK and p38 polyclonal antibodies (Cell Signaling Technology, Beverly, MA), phospho-I$\kappa$B$\alpha$ (Ser32/Ser36), phospho-Akt (Ser473), and Akt polyclonal antibody (Upstate Biotechnology, Inc., Lake Placid, NY) overnight at 4 °C. The membranes were subsequently probed with antimouse or antirabbit IgG antibody conjugated to horseradish peroxidase (Transduction Laboratories, Lexington, KY) and visualized using enhanced chemiluminescence (ECL, Amersham). The densities of the bands were quantitated with a computer densitometer (AlphaImager 2200 System). All of the membranes were stripped and reprobed for $\beta$-actin (Sigma Chemical Co.) as the loading control.

**PCNA Immunohistochemistry.** For indirect PCNA immunochemistry, the deparaffinized skin sections (4 $\mu$m) were incubated with 1.2% $H_2O_2$ in PBS to quench the endogenous peroxidase activity. The primary antibody of proliferating cell nuclear antigen (Santa Cruz Biotechnology, Santa Cruz, CA) was diluted 100 times then applied to each section overnight at 4 °C. After washing with PBS, the sections were incubated with a biotin-conjugated horseradish peroxidase antibody (1: 200; Vector, USA) for 1 h at room temperature. Finally, the peroxidase was detected using the 3,3-diaminobenzidine tetrahydrochloride reaction, which produced the brown label in the epidermal tissue. The numbers of PCNA-positive staining cells were counted in six different fields (×200) at both ends as well as in the middle for each section. The PCNA index was expressed as the average number of stained cells per field divided by the total number of dermal cells and multiplied by 100.

**Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction.** The gene expression was detected as described previously (*18*). Briefly, total RNA of epidermal skin was extracted using Trizol reagent according to the supplier's protocol. A total of 2 $\mu$g of RNA was transcribed into cDNA using SuperScript II RNase H-reverse transcriptase (Invitrogen, Renfrewshire, U.K.) in a final volume of 20 $\mu$L.

In the real-time PCR analysis, specific primers and a fluorogenic probes were designed to target the conserved regions of various genes using the LightCycler (LC) probe design software (Roche, USA), according to the manufacturer's guidelines for the design of PCR primers and TaqMan probes. TaqMan probes used in this experiment are as follows: iNOS, 5′-ACCCTAAGAGTCACCAAAATGG-3′ and 5′-CCAGGGATTCTG-GAACATTCT-3′; COX-2, 5′-GGGAGTCTGGACATTGTGAA-3′ and 5′-GCACGTTGATTGTAGGTGGACTGT-3′; $\beta$-actin, 5′-CCAACCG-TGAAAAGATGACC and 5′-ACCAGAGGCATACAGGGACA.

The thermal cycling conditions are 5 min at 94 °C, followed by 45 cycles, in which each cycle was at 94 °C for 15 s and at 60 °C for 1 min. The relative expression level of the gene in samples was calculated with the LightCycler software, normalized with the housekeeping control ($\beta$-actin).

**Preparation of Cytosolic and Nuclear Extracts from Mouse Skin.** Cytosolic and nuclear protein extraction was performed as previously described (*18*). Briefly, the epidermal samples were extracted by homogenization in 0.5 mL of ice-cold hypotonic buffer A, then homogenized in Polytron for 1 min. The homogenates were incubated on ice under gentle shaking for 15 min. After centrifugation at 1,000 rpm for 5 min to remove tissue debris, the supernatant constituted the cytosolic fraction. The nuclear pellet was resuspended in 200 $\mu$L of high salt extraction buffer C and kept on ice for 30 min, then centrifuged at 14,000 rpm for 5 min. The supernatant containing nuclear proteins was transferred into a new tube and stored at −70 °C after the determination of protein concentration by using a protein assay kit (Bio-Rad, São Paulo, Brazil).

**Electrophoretic Mobility Shift Assay (EMSA).** Nuclear protein extraction was as described above. The EMSA analysis was performed by using a nonradioactive (biotin label) gel shift assay according to the manufacturer's protocol. The NF-$\kappa$B consensus oligonucleotide probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was end-labeled with biotin (Pierce, Rockford, IL) with terminal deoxynucleotidyl transferase (TdT). For the binding reaction, 6 $\mu$g of nuclear extract protein was incubated in a total volume of 20 $\mu$L with binding buffer containing 50 fmol of biotin end-labeled oligonucleotide. The mixture was further incubated at room temperature for 20 min. The specificity was determined by adding a 100-fold excess of unlabeled double-stranded consensus oligonucleotide to the reaction mixture to act as a competition reaction. Following the addition of 5 $\mu$L of sample buffer, the DNA−protein complexes were resolved on a 6% nondenaturing polyacrylamide gel in a 0.5× TBE buffer at 100 V for 2 h then transferred to nylon membrane. Finally, the biotin-labeled DNA was detected by chemiluminescence using the LightShift Chemiluminescent EMSA kit (Pierce) and exposed to X-ray film.

**Two-Stage Tumorigenesis in Mouse Skin.** The antitumor promoting activity of magnolol was examined by a standard initiation−promotion with DMBA and TPA, as reported previously (*18*). One group was composed of 12 female ICR mice. These mice were given commercial rodent pellets and fresh tap water ad libitum, both of which were changed twice a week. The dorsal region of each mouse was shaved with an electric clipper 2 days before initiation. Mice at 6 weeks old were started on 200 nmol of DMBA in 200 $\mu$L of acetone, and control mice received 200 $\mu$L of acetone alone. One week after initiation, the mice were topically

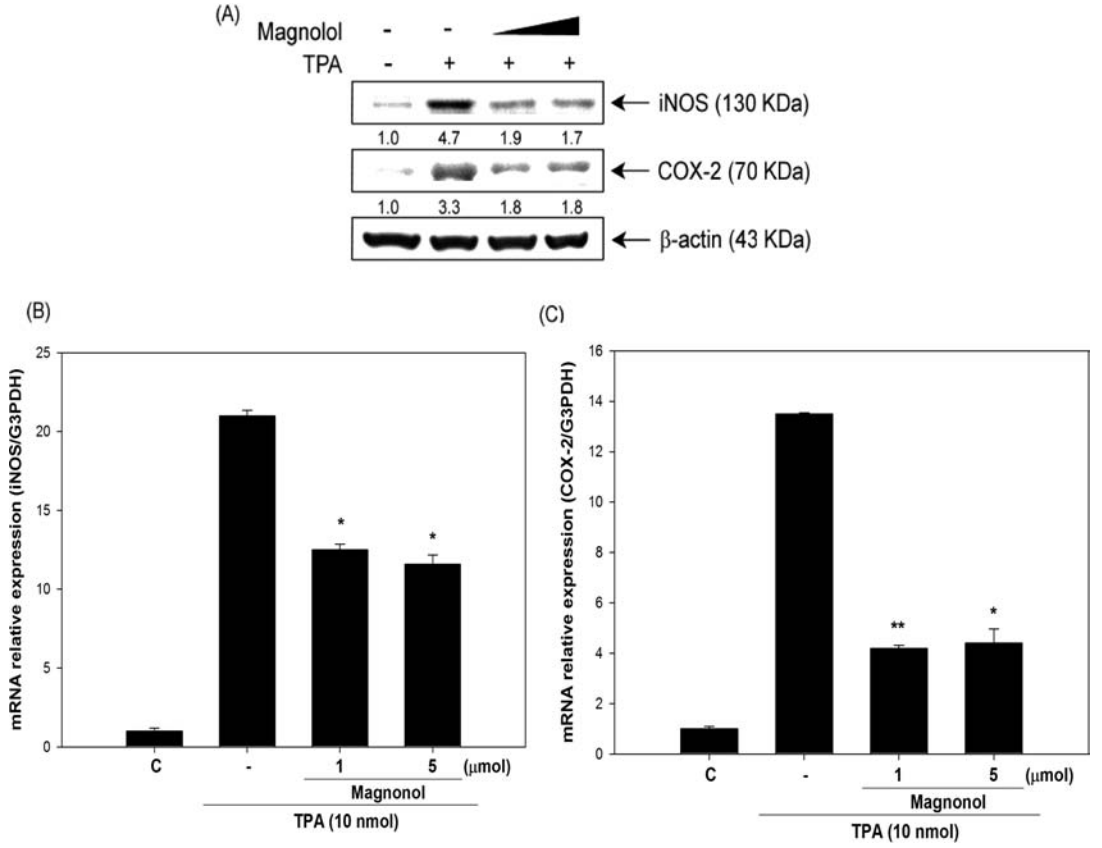


**Figure 2.** Inhibitory effects of magnolol on TPA-induced iNOS and COX-2 protein expression. (**A**) Mice were treated topically with 0.2 mL of acetone or magnolol (1 and 5 $\mu$mol) in the same volume of acetone 30 min prior to 10 nmol TPA, and animals were killed 2 and 4 h respectively after the TPA treatment. The epidermal proteins were analyzed for iNOS and COX-2 by Western blotting analysis. The Western blot is representative of at least three independent experiments. Quantification of iNOS and COX-2 expression were normalized to $\beta$-actin using a densitometer. (**B**) Effect of *iNOS* and (**C**) *COX-2* gene expression on topical application of TPA in mouse skin. Female ICR mice were treated topically with acetone alone or 10 nmol TPA on dorsal skins for the indicated time periods. Mice were treated topically with 0.2 mL of acetone or magnolol (1 and 3 $\mu$mol) in the same volume of acetone 30 min prior to 10 nmol TPA, and animals were killed 1 and 2 h, respectively, after the TPA treatment. Two micrograms of cDNA was subject to real-time PCR. The mRNA expressions of iNOS and COX-2 genes were performed using the LightCycler System and TaqMan probe real-time polymerase chain reaction. Data are mean $\pm$ SE, *, $P < 0.05$, **, $P < 0.01$, and were vs TPA alone.

treated with 200 $\mu$L of acetone or promoted with TPA (5 nmol in 200 $\mu$L of acetone) twice a week for 20 weeks. For the other two groups, the mice were treated with magnolol (1 and 5 $\mu$mol in 200 $\mu$L of acetone) 30 min before each TPA treatment. Tumors of at least 1 mm of diameter in an electronic digital caliper were counted and recorded twice every week, and the diameters of skin tumors were measured at the same time. The results were expressed as the average number of tumors per mouse, percentage of tumor-bearing mice, and size distribution per mouse.

**Statistical Analysis.** Data are presented as the means $\pm$ SE for the indicated number of independently performed experiments. One-way Student's *t*-test was used to assess the statistical significance between the TPA- and magnolol plus TPA-treated groups. A *P*-value $< 0.05$ was considered statistically significant.

## RESULTS

**Inhibitory Effects of Magnolol on TPA-Induced iNOS and COX-2 Expression in Mouse Skin.** The anti-inflammatory activity of magnolol can be demonstrated by its effect on iNOS and COX-2 expression in TPA-stimulated mouse skin. When TPA was applied topically on the shaved area (backs) of female ICR mice, the levels of iNOS and COX-2 proteins were increased, with maximal expression observed at 2 and 4 h, respectively (**Figure 2A**). As shown in **Figure 2A**, topical application of magnolol, 30 min prior to TPA treatment resulted in a dramatic reduction in the levels of iNOS and COX-2 proteins in a dose-dependent manner in mouse skin. To investigate whether or not magnolol has any influence on TPA-induced *iNOS* and *COX-2* gene expression, we applied magnolol at 1 or 5 $\mu$mol, 30 min prior to TPA treatment. From the result of this experiment, we have found that there is a statistically significant ($P < 0.01$) suppression of *iNOS* and *COX-2* gene expression in a dose-dependent manner in mouse skin (**Figure 2B** and **C**).

**Inhibitory Effect of Magnolol on TPA-Induced Activation of NF$\kappa$B and Phosphorylation and Degradation of I$\kappa$B$\alpha$ Protein Expression.** Since iNOS and COX-2 are frequently regulated by activating the NF$\kappa$B signaling pathway (*19*, *20*) and NF$\kappa$B activation and nuclear translocation are preceded by the phosphorylation and proteolytic degradation of I$\kappa$B$\alpha$ (*21*), it is of importance to investigate the inhibitory activity of magnolol against the activation and nuclear translocation of p65 and p50, the functional active subunit of NF$\kappa$B in mouse skin. We determined the phosphorylation and cytoplasmic levels of I$\kappa$B$\alpha$ protein expression by immunoblot analysis to differentiate the potential attributes due to the inhibitory effect of magnolol and its effect on I$\kappa$B$\alpha$ degradation. By topical application of magnolol 30 min prior to TPA treatment, the inhibition of TPA-induced degradation and phosphorylation of I$\kappa$B$\alpha$ protein exhibited a dose-dependent manner (**Figure 3A**). The effect of magnolol treatment on TPA-induced NF$\kappa$B-DNA binding activity was evaluated using the electrophoretic mobility shift assay (EMSA) (**Figure 3B**), and it was found that magnolol suppressed TPA-induced NF$\kappa$B-DNA

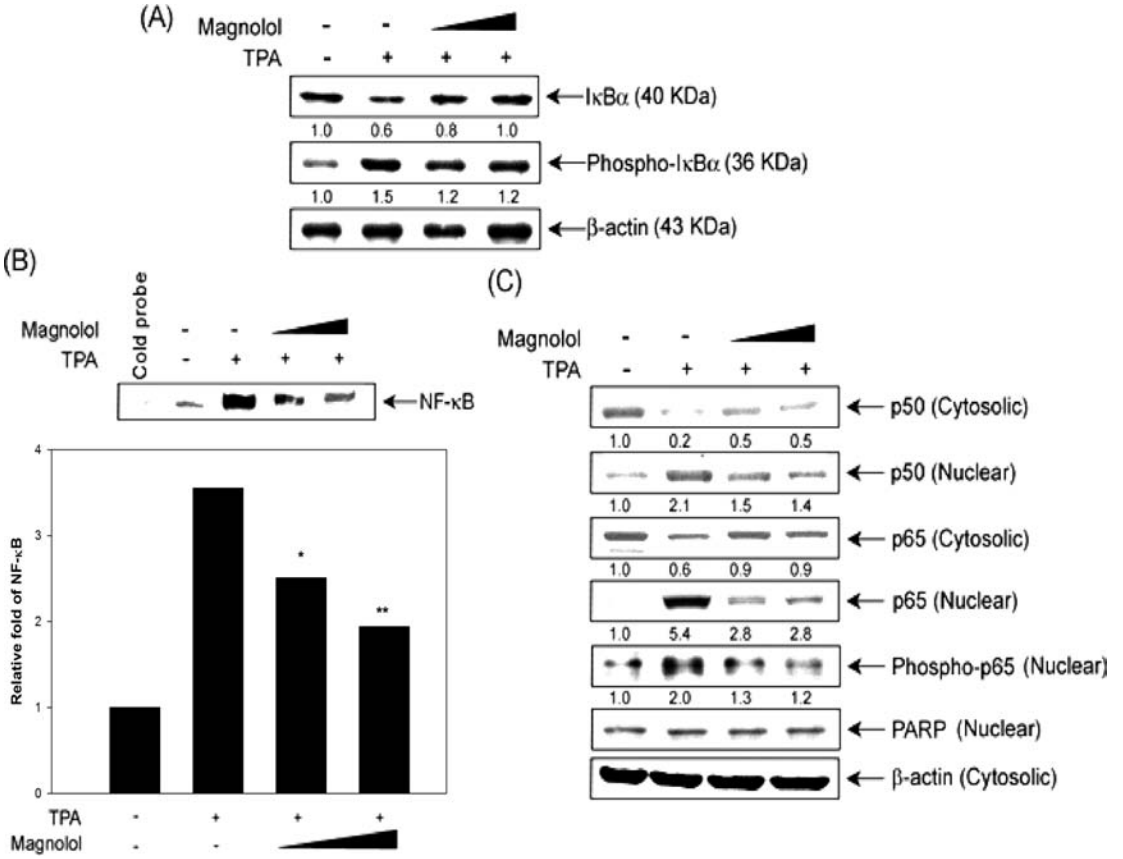


**Figure 3.** Effect of magnolol on TPA-induced NF-$\kappa$B activation. (**A**) Mice were treated topically with 0.2 mL of acetone or magnolol (1 and 5 $\mu$mol) in the same volume of acetone 30 min prior to 10 nmol TPA. All mice were killed 1 h after the TPA treatment, and nuclear and cytosolic extracts from mouse skin were assayed for p-I$\kappa$B and I$\kappa$B. (**B**) EMSA analysis was performed by equal 6 $\mu$g of nuclear extracts from mouse epidermis with a biotin-labeled (nonradioactive) NF-$\kappa$B probe. (**C**) p50, p65, p-p65 (Ser 536), PARP, and $\beta$-actin by Western blotting analysis. All analyses were representative of at least three independent experiments. The values under each lane indicate relative density of the band normalized to $\beta$-actin. Data are mean $\pm$ SE, *, $P < 0.05$, **, $P < 0.01$, and were vs TPA alone.

binding activity in mouse skin in a dose-dependent manner. Addition of cold consensus NF$\kappa$B oligonucleotide (100-fold) abolished the mobility shift band, demonstrating the specificity of the protein/DNA interaction. Supershift analysis has shown that induced NF$\kappa$B was a p65/p50 heterodimer. We also found that the induction of NF$\kappa$B-DNA binding activity coincided with the degradation of I$\kappa$B$\alpha$ and nuclear translocation of both p50 and p65/RelA.We next determined the effect of magnolol on inhibiting NF$\kappa$B nuclear translocation. Topical application of magnolol onto mouse skin inhibited TPA-induced NF$\kappa$B nuclear translocation in a dose-dependent manner as shown by the Western blot analysis (**Figure 3C**). The phosphorylation of p65/RelA at serine 536 was also inhibited by the magnolol pretreated animal group in a dose dependent manner (**Figure 3C**, lower panel). In this experiment, PARP, a nuclear protein, and $\beta$-actin, a cytosolic protein, were used as controls to confirm that there was no contamination during the extraction of each fraction.

**Inhibitory Effect of Magnolol on TPA-Induced Phosphorylation of Epidermal MAP Kinases, PI3K/Akt.** Studies have shown that the p38, ERK (p44/42) MAPK, and PI3K/Akt signaling pathway is involved in the TPA-mediated induction of iNOS and COX-2 by diverse mechanisms including the modulation of signaling via NF$\kappa$B and AP-1 (*22*, *23*). Therefore, we investigated the effects of magnolol on the TPA-induced phosphorylation of p38, ERK1/2 MAPK, and PI3K/Akt in mouse skin. Western blot analysis revealed that topical application of TPA alone caused significant increase in the phosphorylation of p38 (3.1-fold increase) and ERK MAPK kinase (1.5-fold) in mouse skin as compared to that

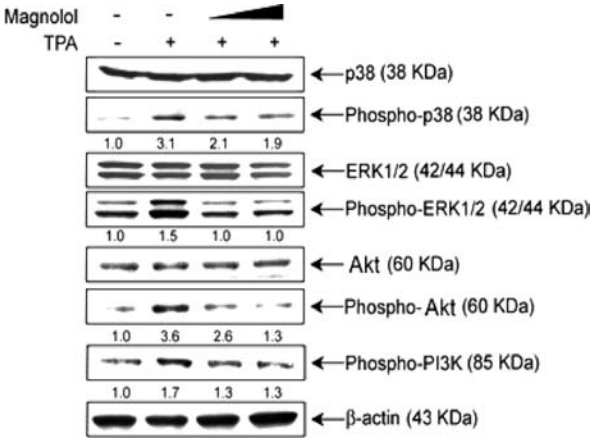


**Figure 4.** Effects of magnolol on TPA-induced activation of MAPK and PI3K/Akt in mouse skin. Mice were treated topically with 0.2 mL of acetone or magnolol (1 and 5 $\mu$mol) in the same volume of acetone 30 min prior to 10 nmol TPA. All mice were killed 1 h after the TPA treatment, and expression of p-p38 and p-ERK1/2 MAPK, p-PI3K, and p-Akt were measured by Western blot analysis. The values under each lane indicate relative density of the band normalized to $\beta$-actin. Data are representative of at least independent experiments, which showed a similar result.

in the vehicle-treated controls. However, pretreatment of magnolol retarded the phosphorylation of p38 and ERK1/2 MAPK kinase in TPA-treated mouse skin (**Figure 4**). We also assessed

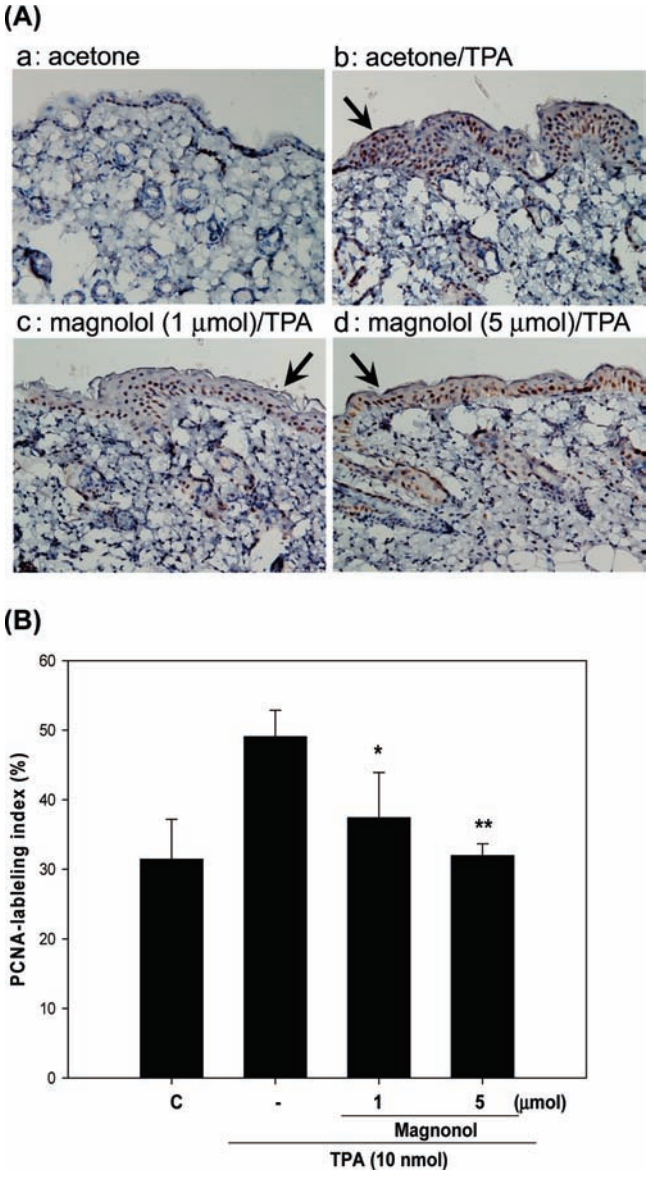


**Figure 5.** Inhibitory effects of magnolol on TPA induction of PCNA-positive cells in mouse skin. (**A**) All mice were sacrificed at 24 h after the TPA treatment for PCNA immuno-staining as described in Materials and Methods. (**B**) PCNA-labeling index (%) was counted from 6 different fields (×200) from each mouse. Data are mean ± SE *, $P < 0.05$, **, $P < 0.01$, and were vs TPA alone.

whether or not PI3K/Akt signaling is involved in cellular responses to TPA by performing Western blot analysis with antibody in the phosphorylated form of PI3K and Akt. Densitometric analysis of blots revealed significant increase in the phosphorylation of PI3K and Akt in mouse skin treated with TPA. We observed that preapplication of magnolol prior to TPA treatment attenuated TPA-induced phosphorylation of PI3K and Akt in mouse skin (**Figure 4**). More importantly, no changes were observed in the total epidermal Akt content in mice treated with both TPA and magnolol as compared to that in the vehicle-treated control. These results of our immunoblot analyses suggest that the inhibition of iNOS and COX-2 expression by magnolol might block TPA-induced NF$\kappa$B activation by inhibiting the p38, ERK1/2 MAPK, and PI3K/Akt pathway, which interrupts the degradation of I$\kappa$B$\alpha$.

**Antiproliferative Activities of Magnolol in Mouse Skin.** PCNA (proliferating-cell nuclear antigen) forms a ring around DNA to

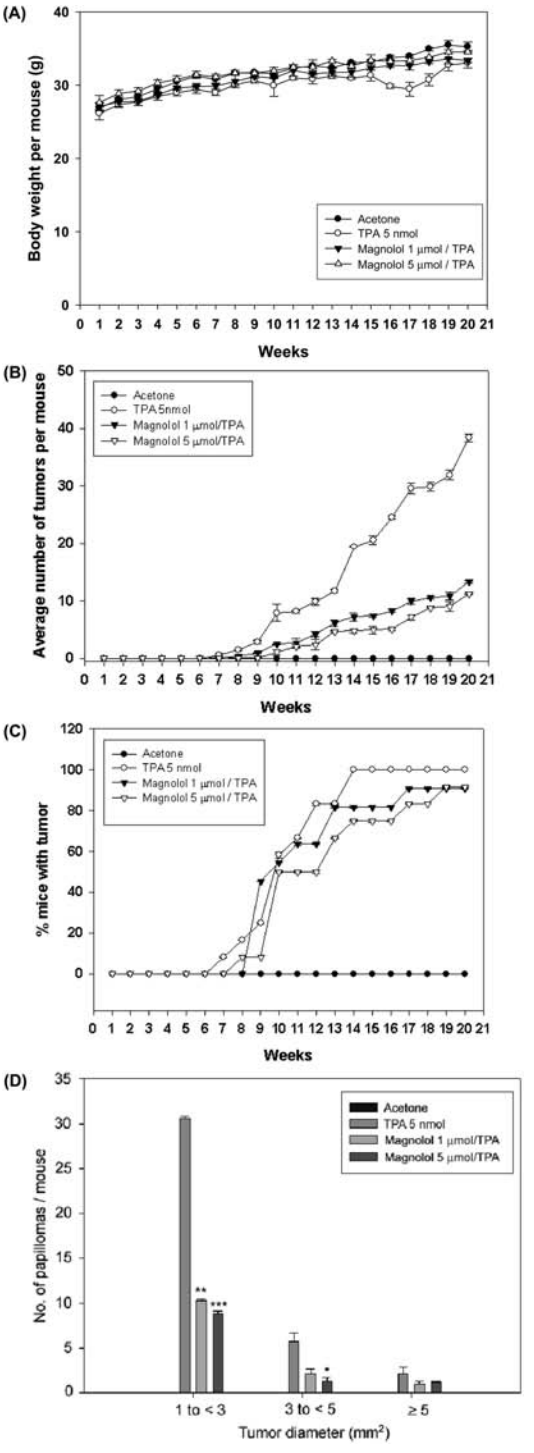


**Figure 6.** Antitumor promoting effects of magnolol on DMBA/TPA-induced skin tumorigenesis in ICR mice. Tumor promotion in all mice was initiated with DMBA (200 nmol) and promoted with TPA (5 nmol) twice weekly, starting 1 week after initiation. Magnolol (1 and 5 $\mu$mol) was dissolved in 0.2 mL of acetone and topically applied 30 min prior to each TPA treatment. During the experiment of tumor promotion, the diameters of skin tumors were measured by an electronic digital caliper twice every week. Tumors of at least 1 mm in diameter were counted and recorded weekly, as described in Materials and Methods. (**A**) Body weight of mice. (**B**) Average number of tumors per mouse (tumor multiplicity). (**C**) Percentage of tumor-bearing mice (tumor incidence). (**D**) The tumor size was recorded as the average of length × width (millimeter square) per mouse. *, $P < 0.05$, **, $P < 0.01$, and ***, $P < 0.001$ indicate statistically significant differences from the TPA-treated group. Statistical analysis was done by Student's *t*-test.

facilitate and control DNA replication. The crucial involvement of PCNA in cellular proliferation and its tight association with cancer transformation resulted in the frequent use of PCNA as a marker for cell proliferation (*24*). Upon treatment with TPA for 4 h, the expression of PCNA increased mainly in the epidermal layer, which was suppressed by magnolol pretreatment (**Figure 5A**). Pretreatment with 1 and 5 μmol of magnolol prior to TPA application significantly reduced PCNA-labeling indices by 23.7 and 34.9%, respectively (**Figure 5B**).

**Antitumor-Promoting Activity of Magnolol in Mouse Skin.** Upregulation of iNOS and COX-2 occurs in many pathological conditions, such as in tumorigenesis. Since application of 1 and 5 μmol of magnolol to mouse skin significantly inhibited various molecular targets that play significant roles in the progression of skin tumors, we selected these doses for assessing the antitumor-promoting potential of magnolol in 7,12-dimethylbenz(*a*)anthracene-initiated mouse skin. As shown in **Figure 6**, with DMBA and TPA treatment, the tumor incidence in this positive control group was 100% 20 weeks after promotion. In contrast, administration of DMBA followed by repeated application of acetone produced no tumors. Throughout the experiment, there was no noticeable difference in weight gain between the mice treated with two doses of magnolol and with those not treated, indicating that the topical application of magnolol did not cause any toxicity (**Figure 6A**). The average number of tumors per mouse in the control was 37.0 at the end of the 20 week experiment. In the treated groups, pretreatment with magnolol dose-dependently reduced the number of tumors per mouse by 65.8% and 71.1% at 1 μmol dose and 5 μmol dose, respectively (**Figure 6B**).With magnolol (1 or 5 μmol)-pretreated groups (30 min before TPA), the incidence was reduced by 10% (**Figure 6C**). The tumor promotion data were analyzed in terms of size distribution of papillomas and compared with the positive control group. The number of papilloma (1 to $<3$ mm in diameter) per mouse was dose-dependently inhibited in the magnolol treated group (**Figure 6D**). In addition, magnolol treatment showed a significant inhibition in tumors at sizes of 3 to $<5$ mm groups of mice compared with the positive control group. The animals started on DMBA and treated twice weekly with 5 μmol of magnolol were devoid of any skin tumors throughout the experiment (data not shown), suggesting that magnolol itself is not a tumor promoter.

## DISCUSSION

Chemoprevention is an active cancer preventive strategy to inhibit, delay, or reverse human carcinogenesis. COX-2 and iNOS are important enzymes involved in mediating the inflammation process, cell proliferation, and skin tumor promotion (*25*, *26*). In the current study, our data clearly demonstrate that application of magnolol before TPA treatment affords significant inhibition of TPA-induced iNOS and COX-2 mRNA and protein expression in a dose-dependent manner (**Figure 2**). These inhibitory effects of magnolol in the suppression of TPA-mediated responses in mouse skin suggest that the primary effect of magnolol may be against inflammatory responses, which may then result in the inhibition of tumor promotion.

Activation of NFκB is necessary for TPA induction of the iNOS and COX-2 promoter (*27*). In our study, magnolol was found to inhibit TPA-induced DNA binding activity of NFκB by suppressing the phosphorylation of IκBα and p65 and subsequent nuclear translocation of p50 and p65/RelA subunits of NFκB (**Figure 3**).

Both NFκB and PI3K/Akt signaling pathways have emerged as promising molecular targets in the prevention of cancers. Many signaling pathways, including PI3K/Akt and mitogen-activated protein kinase (MAPK), have been proposed to respond to TPA stimulation (*28*). PI3K activation leads to phosphorylation of phosphatidylinositides, which then activates the downstream main target, Akt, which appears to play various important roles in regulating cellular growth, differentiation, adhesion, and inflammatory reactions (*29*). Since magnolol can significantly inhibit the induction of iNOS and COX genes and proteins, we have investigated whether or not magnolol exerts any influence on or interferes with the signaling molecules, in turn regulating them. In this study, we clearly demonstrated that topical application of TPA resulted in the activation of p38, ERK1/2, and PI3K/Akt. Topical application of magnolol prior to TPA application to mouse skin resulted in the reduction of TPA-induction phosphorylation of p38, ERK1/2 MAPK, and PI3K/Akt in mouse skin (**Figure 4**).

As predicted by the suppressive efficacies of biochemical markers related to inflammation, topical application of magnolol at doses of 1 μmol and 5 μmol, before TPA treatment during the tumor promotion process, significantly lowered the number and size of papillomas. Magnolol inhibited TPA-induced formation of the average number of skin tumors per mouse in a dose-dependent manner. The possible mechanism is that magnolol downregulates inflammatory iNOS and COX-2 gene expression in mouse skin by inhibiting the activation of NFκB and by interfering with the activation of MAPK and PI3K/Akt. Magnolol seems to act as a modulating agent in multiple signaling pathways. We conclude that magnolol has great potential as a novel chemopreventive agent to be used in the treatment of inflammation-associated tumorigenesis.

## ABBREVIATIONS USED

iNOS, inducible nitric oxide synthase; COX-2, cycoloxygenase-2; NF-κB, nuclear factor-κB; AP-1, activator protien-1; MAPK, mitogen-activated protien kinase; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; DMBA, 7,12-dimethylbenz(*a*)anthracene; ICR, Institute of Cancer Research; ERK, extracellular signal-regulate kinase; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; PI3K, phosphatidylinositol 3-kinase.

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