RESEARCH ARTICLE

6-Shogaol is more effective than 6-gingerol and curcumin in inhibiting 12-*O*-tetradecanoylphorbol 13-acetate-induced tumor promotion in mice

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We previously reported that 6-shogaol strongly suppressed lipopolysaccharide-induced overexpression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) in murine macrophages. In this study, we further compared curcumin, 6-gingerol, and 6-shogaol's molecular mechanism of action and their anti-tumor properties. We demonstrate that topical application of 6-shogaol more effectively inhibited 12-*O*-tetradecanoylphorbol 13-acetate (TPA)-stimulated transcription of iNOS and COX-2 mRNA expression in mouse skin than curcumin and 6-gingerol. Pretreatment with 6-shogaol has resulted in the reduction of TPA-induced nuclear translocation of the nuclear factor-κB subunits. 6-Shogaol also reduced TPA-induced phosphorylation of IκBα and p65, and caused subsequent degradation of IκBα. Moreover, 6-shogaol markedly suppressed TPA-induced activation of extracellular signal-regulate kinase1/2, p38 mitogen-activated protein kinase, JNK1/2, and phosphatidylinositol 3-kinase/Akt, which are upstream of nuclear factor-κB and AP-1. Furthermore, 6-shogaol significantly inhibited 7,12-dimethylbenz[*a*]anthracene/TPA-induced skin tumor formation measured by the tumor multiplicity of papillomas at 20 wk. Presented data reveal for the first time that 6-shogaol is an effective anti-tumor agent that functions by down-regulating inflammatory iNOS and COX-2 gene expression in mouse skin. It is suggested that 6-shogaol is a novel functional agent capable of preventing inflammation-associated tumorigenesis.

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1 Introduction

Curcumin (diferuloylmethane), a dietary pigment from *Curcuma longa* L., gives the golden-yellow color and unique flavor to curry. The anti-carcinogenic effects of this compound are demonstrated by its ability to inhibit tumor initiation by azoxymethane, benzpyrene, and 7,12-dimethylbenz[*a*]anthracene (DMBA), and to suppress tumor promotion by phorbol esters. Ginger (*Zingiber officinale*) has been widely used as a spice, and as a medicinal herb in traditional herbal medicine. It is a natural dietary ingredient with antioxidative, anti-inflammatory, and anticarcinogenic properties [1, 2]. Ginger contains several pungent constituents such as gingerols, shogaols, paradols, and gingerdiols. In the fresh ginger rhizome, gingerols were identified as the major active components, and 6-gingerol is the most abundant constituent. The amount of 6-gingerol in fresh

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Abbreviations: COX-2, cyclooxygenase-2; **DMBA,** 7,12-dimethylbenz[*a*]anthracene; **ERK,** extracellular signal-regulate kinase; **ICR,** Institute of Cancer Research; **iNOS,** inducible nitric oxide synthase; **MAPK,** mitogen-activated protien kinase; **NFκB,** nuclear factor-κ; **PI3K,** phosphatidylinositol 3-kinase; **TPA,** 12-*O*-tetradecanoylphorbol 13-acetate

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rhizomes of ginger cultivated in China and Japan was approximately 0.3–0.5% [3]. The shogaols, another homologous series that can be formed by dehydration at the C-5 and C-4 of gingerols during storage or thermal processing, are predominant pungent constituents in the ginger oleoresin from dried ginger [4–6]. It has been reported that the ratio of 6-gingerol to 6-shogaol was about 10:1 in fresh ginger and about 1:1 in dried ginger [3–6].

Topical application of -gingerol onto shaven backs of female Institute of Cancer Research (ICR) mice prior to each topical dose of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) significantly inhibited DMBA-induced skin tumor incidence and tumor burden [7]. It is well established that inflammation is causally linked to carcinogenesis and acts as a driving force in pre-malignant and malignant transformation of cells [8, 9]. Topical application of TPA to mice leads to edema and promotes DMBA-initiated mouse papilloma formation by enhancing inducible nitric oxide synthase (iNOS), COX-2, and ODC protein expression [10]. Specific iNOS and COX-2 inhibitors are able to counteract these biological events [11]. Activated nuclear factor-κB (NFκB) and AP-1 often facilitate transcription of numerous genes, including iNOS and COX-2, resulting in inflammation and tumorigenesis. TPA activates NFκB through a cascade of events that activate inhibitor κB (IκB) kinases (IKKs), which, in turn, phosphorylate IκB. The subsequent ubiquitination and proteasomal degradation of IκB free NFκB to translocate to the nucleus [12]. These kinases can be activated through phosphorylation by upstream kinases, including NFκB-inducing kinase and mitogen-activated protein kinase (MAPK) [13, 14]. In addition, many studies have confirmed the role of cytokines in the induction of transcriptional activity of NFκB through ERK1/2 (p42/44), p38 MAPK, and phosphatidylinositol 3-kinase (PI3K)/Akt pathways [15].

We previously reported that 6-shogaol (Fig. 1) was found to be more potent anti-inflammatory agent compared with 6-gingerol [16], however, the exact molecular mechanisms underlying the anti-inflammatory effect of 6-shogaol in mice remain largely unresolved. In this study, we have evaluated the effect of 6-shogaol on TPA-induced tumor promotion on mouse skin following TPA application, and investigated the inhibitory effect of 6-shogaol on mouse skin tumor promotion using a two-stage skin carcinogenesis model including tumor incidence, multiplicity, and volume. We have also evaluated the effects of the related compounds 6-gingerol and curcumin as potential inhibitors of tumor promotion and their molecular mechanism of action.

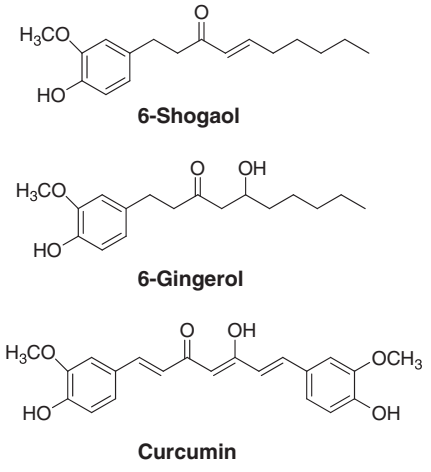

Figure 1. Chemical structures of curcumin, 6-gingerol, and 6-shogaol.

2 Materials and methods

2.1 Chemicals

The isolation and purification of 6-gingerol and 6-shogaol have been described previously [16]. TPA and DMBA were purchased from Sigma Chemical (St. Louis, MO). All other chemicals used were in the purest form available commercially.

2.2 Animals

All animal experimental protocol used in this study was approved by Institutional Animal Care and Use Committee of the National Kaohsiung Marine University (IACUC, NKMU). Female ICR mice at 5–6 wks old were obtained from the BioLASCO Experimental Animal Center (Taiwan, BioLASCO, Taipei, Taiwan). All animals were housed in a controlled atmosphere ($25 \pm 1^{\circ}$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 compounds. Curcumin, 6-gingerol, 6-shogaol, and TPA were dissolved in 200 μL of acetone and applied topically to the shaved area of each mouse.

2.3 Western blot analysis

The 6-week-old female ICR mice were topically treated on their shaved backs with curcumin, 6-gingerol, or 6-shogaol in 200 μL of acetone and 30 min prior to 10 nmol TPA in 200 μL of acetone treatment. The mice were sacrificed by cervical dislocation at the indicated time. Dorsal skins of mice from different experiments were excised for protein isolation. Briefly, skin was immediately excised from the entire torso and the epidermis and dermal fractions were separated by heat treatment (60°C for 30 s). The epidermis was gently removed using a scalpel on ice and the separated skin fractions were immediately placed in liquid nitrogen [17, 18]. Epidermal protein was homogenized on ice for 15 s with a Polytron tissue homogenizer and lysed in 0.5 mL ice-cold lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM NaF, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethanesulfonyl

fluoride, 1% NP-40, and 10 μg/mL leupeptin) on ice for 30 min, followed by centrifugation at 10 000 × *g* for 30 min at 4°C. The cytosolic fraction (supernatant) protein was measured by Bio-Rad protein assay (Bio-Rad Laboratories, Munich, Germany). The samples (50 μg of protein) were mixed with 5 × sample buffer containing 0.3 M Tris-HCl (pH 6.8), 25% 2-mercaptoethanol, 12% SDS, 25 mM EDTA, 20% glycerol, and 0.1% bromophenol blue. The mixtures were boiled at 100°C for 5 min and were loaded to a stacking gel and then resolved by 12% SDS–polyacrylamide minigels at a constant current of 20 mA. Proteins on the gel were electro-transferred onto a 45 μ immobile membrane (polyvinylidene difluoride; Millipore, Bedford, MA, USA) with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membranes were blocked with blocking solution (20 mM Tris-HCl pH 7.4, 0.2% Tween 20, 1% bovine serum albumin, and 0.1% sodium azide) and probed overnight at 4°C with primary antibody (diluted 1:1000 in blocking solution). The primary antibodies used were as follows: iNOS, IκBα, p50, p65, and phospho-PI3K(Tyr508) polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA), COX-2 monoclonal antibodies (Transduction Laboratories, BD Biosciences, Lexington, KY, USA), phospho-p65 (Ser536), phospho-p38 (Thr180/Tyr182), phospho-ERK1/2 (Thr202/Tyr204), ERK, and p38 polyclonal antibodies (Cell Signaling Technology, Beverly, MA, USA), phospho-IκBα (Ser32/Ser36), phospho-Akt (Ser473), Akt polyclonal antibodies (Upstate Biotechnology, Lake Placid, NY, USA). The membranes were subsequently probed with anti-mouse or anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase (Transduction Laboratories) and visualized using enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK). The densities of the bands were quantified with a computer densitometer (AlphaImager™ 2200 System). All the membranes were stripped and re-probed for β-actin (Sigma Chemical) as loading control.

2.4 Quantitative real-time reverse-transcription PCR

Total RNA from epidermal skin was extracted using TRIZOL reagent according to the supplier's protocol. The RNA concentration was determined by measuring the ultraviolet absorbance 260 and 280 nm and the RNA was stored at −70°C until real-time PCR analysis. A total of 2 μg RNA was transcribed into cDNA using SuperScript II RNAse H-reverse transcriptase (Invitrogen, Renfrewshire, UK) in a final volume of 20 μL. Reverse transcription reactions were performed at 42°C for 50 min and 99°C for 5 min in a LightCycler® 1.5 System (Roche, Indianapolis, IN). Negative controls were simultaneously performed with all of the components except reverse transcription.

In the real-time PCR analysis, specific primers, and fluorogenic probes were designed to target the conserved regions of various genes using the Lightcycler probe design software (Roche), according to the manufacturer's guidelines for the design of PCR primers and TaqMan probes. The PCR primers and TaqMan probes used in this experiment are as follows: iNOS, 5′-ACCCTAAGAGTCACCAAAATGG-3′ and 5′-CCAGGGATTCTGGAACATTCT-3′; COX-2, 5′-GGGAGTCTGGACATTGTGAA-3′ and 5′-GCACGTTGATTGTAGGTGGACTGT-3′; β-actin, 5′-CCAACCGTGAAAAGATGACC and 5′-ACCAGAGGCATACAGGGACA.

All TaqMan PCR primers were located in two different exons of each gene to avoid amplification of any contaminating genomic DNA. All PCR reactions were performed using the LightCycler System (Roche) in a total volume of 20 μL containing 1 × *Taq* polymerase buffer, 5 mmol/L $MgCl_2$, 200 μmol/L deoxynucleotides, 300 nmol/L each primer, 150 nmol/L probe, 1 U *Taq* polymerase and 20 ng complementary DNA (cDNA). For the negative controls, water was used instead of cDNA. Gene amplification was done in duplicate for each sample. The thermal cycling conditions were 5 min at 94°C, followed by 45 cycles of 94°C for 15 s and at 60°C for 1 min. The relative expression levels of the genes in each sample were calculated with the LightCycler software and normalized with a housekeeping control (β-actin).

2.5 Preparation of cytosolic and nuclear extracts from mouse skin

Cytosolic and nuclear protein extraction was performed as previously described [19]. In brief, the skins were washed with cold water and the epidermal cells from the dorsal skin of mice were stripped off. The epidermal samples were extracted by homogenization in 0.5 mL of ice-cold hypotonic buffer A containing 10 mM *N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM ethlenediaminetetraacetic acid, and 0.1 mM phenylmethylsulfonylfluoride, and then homogenized in a Polytron for 1 min. The homogenates were incubated on ice with gentle shaking for 15 min and centrifuged at 1000 rpm for 5 min to remove tissue debris. The supernatant contained the cytosolic fraction. The pellet was resuspended in buffer A supplemented with 50 μL of 10% Nonidet P-40 (NP-40), vortexed, and centrifuged for 2 min at 14 000 rpm. The nuclear pellet was resuspended in 200 μL of high salt extraction buffer C (50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 10% glycerol). It was kept on ice for 30 min and then centrifuged at 14 000 rpm for 5 min. The supernatant constituted nuclear proteins was transferred into a new tube and stored at −70°C after determination of protein concentration with a protein assay kit (Bio-Rad, São Paulo, Brazil).

2.6 Two-stage tumorigenesis in mouse skin

The anti-tumor promoting activity of curcumin, 6-gingerol, or 6-shogaol was examined by a standard initiation–promotion

with DMBA and TPA, as reported previously [20]. 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. Six-week-old mice were treated with 200 nmol DMBA in 200 μL of acetone. Control mice received 200 μL of acetone alone. One week after initiation, the mice were topically treated with 200 μL of acetone or 5 nmol TPA in 200 μL acetone twice a week for 20 wk. For the other groups, the mice were treated with curcumin, 6-gingerol, or 6-shogaol (2.5 μmol in 200 μL 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 tumor size distribution *per* mouse.

2.7 Statistical analysis

Data are presented as mean ± standard error for the indicated number of independently performed experiments. A one-way Student's *t*-test was used to assess the statistical significance between the TPA- and curcumin, 6-gingerol, or 6-shogaol plus TPA-treated groups. A p-value < 0.05 was considered statistically significant.

3 Results

3.1 Inhibitory effect of curcumin, 6-gingerol, and 6-shogaol on TPA-induced iNOS and COX-2 expression in mouse skin

The anti-inflammatory activity of curcumin, 6-gingerol, and 6-shogaol can be demonstrated by its effect on iNOS and COX-2 expression in TPA-stimulated mouse skin. We have shown previously that TPA, a mitogen and well-known promoter of skin tumorigenesis, stimulates iNOS and COX-2 expression in mouse skin [20]. 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 (Fig. 2A). As shown in Fig. 2B, topical application of curcumin, 6-gingerol, and 6-shogaol 30 min prior to TPA treatment, resulted in a significant reduction in the levels of iNOS and COX-2 proteins in a dose-dependent manner in mouse skin. Interestingly, 6-shogaol strongly suppressed the expression of iNOS and COX-2 proteins. The data indicated that 6-shogaol was more active than curcumin and 6-gingerol. To investigate whether or not curcumin, 6-gingerol, and 6-shogaol has any influence on TPA-induced *iNOS* and *COX-2* gene expression, we applied curcumin, 6-gingerol, and 6-shogaol at 1 or 2.5 μmol, 30 min prior to TPA treatments. From the result of this experiment, we have found that there is a statistically significant suppression of *iNOS* and *COX-2* gene expression in a dose-dependent manner in mouse skin (Fig. 3).

3.2 Inhibitory effect of curcumin, 6-gingerol, and 6-shogaol on TPA-induced NFκB activation in mouse skin

iNOS and COX-2 are frequently regulated by activating the NFκB signaling pathway [21, 22], and NFκB activation and nuclear translocation are preceded by the phosphorylation and proteolytic degradation of IκBα [23]. Therefore, it is important to investigate how curcumin, 6-gingerol, and 6-shogaol inhibit activation and nuclear translocation of p65 and p50, the functional active subunits of NFκB in mouse skin. We first determined the phosphorylation and cytoplasmic levels of IκBα protein expression by immunoblot analysis to determine the potential contribution of the inhibitory effect of curcumin, 6-gingerol, and 6-shogaol and its effect on IκBα degradation. Topical application of TPA led to phosphorylation and degradation of IκBα, which were significantly repressed by curcumin, 6-gingerol, and 6-shogaol (2.5 μmol) pre-treatments (Fig. 4A). By the topical application of curcumin, 6-gingerol, and 6-shogaol onto mouse skin (prior to TPA application), we found that TPA-induced NFκB nuclear translocation was inhibited by the curcumin, 6-gingerol, and 6-shogaol pre-treated animal groups in a dose-dependent manner (Fig. 4B). In this experiment, PARP, a nuclear protein, and β-actin, a cytosolic protein, were used as controls to confirm that there was no contamination during extraction of each fraction. Additionally, curcumin, 6-gingerol, and 6-shogaol treatments caused a significant reduction of TPA-induced nuclear translocation and phosphorylation of p65/RelA, respectively, the functionally active subunit of NFκB protein (Fig. 4C).

3.3 Inhibitory effect of curcumin, 6-gingerol, and 6-shogaol on TPA-induced activation of MAPKs, and PI3K, Akt/protein kinase B

MAPKs are known to regulate NFκB activation by multiple mechanisms. Studies have shown that the p38, ERK, and PI3K/Akt signaling pathways are involved in the TPA-mediated induction of iNOS and COX-2 by diverse mechanisms, including the modulation of signaling *via* NFκB, AP-1, STATs in mouse skin [24–26]. Therefore, we investigated the effects of curcumin, 6-gingerol, and 6-shogaol on TPA-induced phosphorylation of ERK, p38 MAPK, JNK, and PI3K/Akt in mouse skin. Western blot analysis revealed that topical application of TPA alone caused significant increase in the phosphorylation of ERK (6.2-fold increase), p38 MAPK (6.9-fold), and JNK (11.5-fold) in mouse skin compared with vehicle-treated controls. Pre-treatment of 6-shogaol strongly inhibited the phosphorylation of p38, ERK, and JNK in TPA-treated mouse skin in a dose-dependent manner (Fig. 5A and B). We also assessed

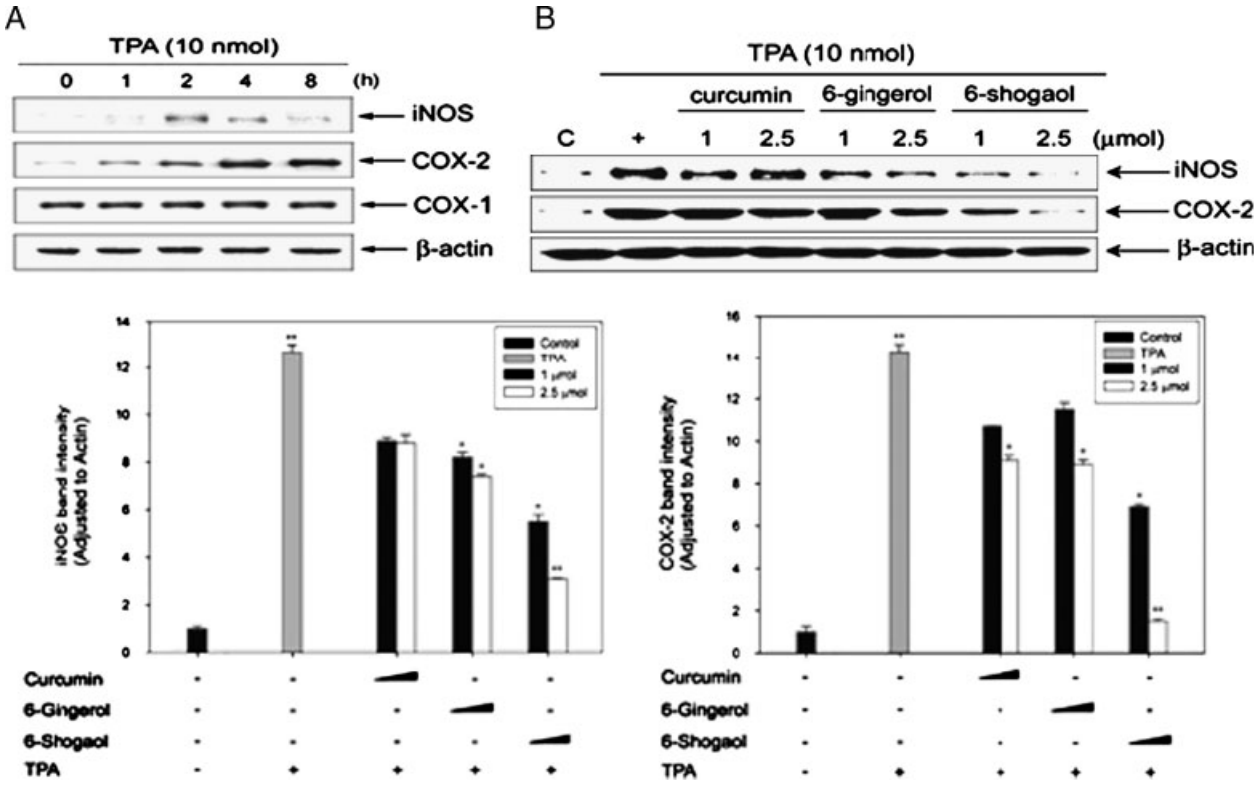

Figure 2. Inhibitory effects of curcumin, 6-gingerol, and 6-shogaol on phorbol ester-induced iNOS and COX-2 protein expression. (A) Time course for iNOS, COX-2, and COX-1 protein expression on topical application of TPA in mouse skin (6 wk of age, six *per* group). Female ICR mice were treated topically with acetone alone or 10 nmol TPA on dorsal skins for indicated time periods. (B) Mice (6 wk of age, six *per* group) were treated topically with 0.2 mL acetone or curcumin, 6-gingerol, 6-shogaol (1 and 2.5 μmol) in the same volume of acetone 30 min prior to 10 nmol TPA, and were sacrificed 2 h (for iNOS) and 4 h (for COX-2), 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 was normalized to β-actin using a densitometer.

whether PI3K/Akt signaling is involved in cellular responses to TPA by performing Western blot analysis with antibody in phosphorylated form of PI3K and Akt. Densitometric analysis of blots revealed significant increases in the phosphorylation of PI3K and Akt in mouse skin treated with a single topical application of TPA. We observed that pre-application of 6-shogaol prior to TPA treatment attenuated TPA-induced phosphorylation of PI3K and Akt in mouse skin (Fig. 5C). The results of these studies indicate that 6-shogaol is more active than curcumin and 6-gingerol. More importantly, no change was observed in the total epidermal Akt content in mice treated with both TPA and compounds compared with vehicle-treated control.

3.4 Effect of curcumin, 6-gingerol, and 6-shogaol on TPA-induced tumor promotion in mouse epidermis

Upregulation of iNOS, COX-2, and ODC occurs in many pathological conditions, such as in tumorigenesis. Since application of 2.5 μmol of 6-shogaol to mouse skin significantly inhibited various molecular targets that play significant roles in the progression of skin tumors, we selected this dose and compared curcumin and 6-gingerol for assessing the anti-tumor promoting potential of 6-shogaol in DMBA-treated mouse skin. As shown in Fig. 6, throughout the experiment, there was no noticeable difference in weight gain between the mice treated with curcumin, 6-gingerol, and 6-shogaol and those not treated, indicating that the topical application of curcumin, 6-gingerol, and 6-shogaol did not cause any toxicity (Fig. 6A). Mice initiated with DMBA and promoted with TPA twice weekly for 20 wk developed an average of 17.0 tumors/mouse. In the treated groups, pre-treatment with curcumin, 6-gingerol, and 6-shogaol reduced the number of tumors *per* mouse by 39.4, 70.6, and 91.2% at 2.5 μmol dose, respectively (Fig. 6B), and the percentage of animals with tumors was decreased by 18, 28, and 58%, respectively (Fig. 6C). Tumor incidence in this positive control group was 100% 20 wk after promotion. In contrast, administration of DMBA followed by repeated application of acetone produced no tumors. The tumor promotion data were analyzed in terms of size distribution of papillomas and compared with the positive control group.

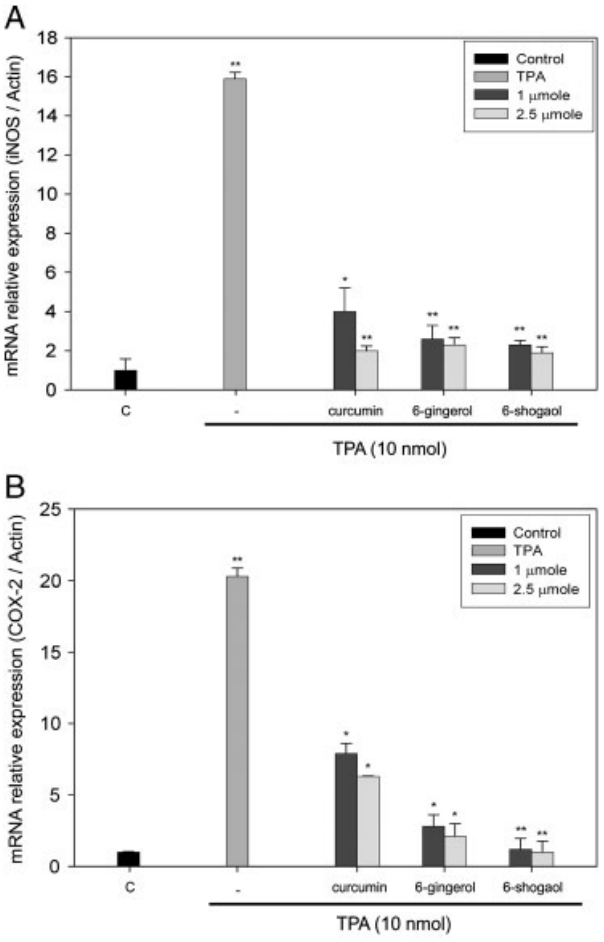

Figure 3. Effects of curcumin, 6-gingerol, and 6-shogaol on *iNOS* and *COX-2* gene expression on topical application of TPA in mouse skin. Mice (6 wk of age, six *per* group) were treated topically with 0.2 mL acetone or curcumin, 6-gingerol, and 6-shogaol (1 and 2.5 μmol) in the same volume of acetone 30 min prior to 10 nmol TPA, and were sacrificed 1 h (for *iNOS*) and 2 h (for *COX-2*), respectively, after the TPA treatment. Two microgram of cDNA were subject to real-time PCR. The mRNA levels of *iNOS* and *COX-2* gene were quantified using the LightCycler System and TaqMan probe real-time PCR. Data are mean ± SE. $^{**}p<0.001$ (control *versus* TPA alone; $^{*}p<0.05$; $^{**}p<0.01$ were verse TPA alone.

The number of papilloma (1 to < 3 mm in diameter) *per* mouse was significantly inhibited in the 6-gingerol and 6-shogaol treated group (Fig. 6D). In addition, curcumin, 6-gingerol, and 6-shogaol treatment showed a significant inhibition in tumors at sizes of 3 to < 5 mm and ≥ 5 mm as is evident in a significant reduction in tumor size compared with the positive control group. The animals started on DMBA and treated twice weekly with 2.5 μmol of curcumin, 6-gingerol, and 6-shogaol were devoid of any skin tumors throughout the experiment (data not shown), suggesting that curcumin, 6-gingerol, and 6-shogaol are not tumor promoters.

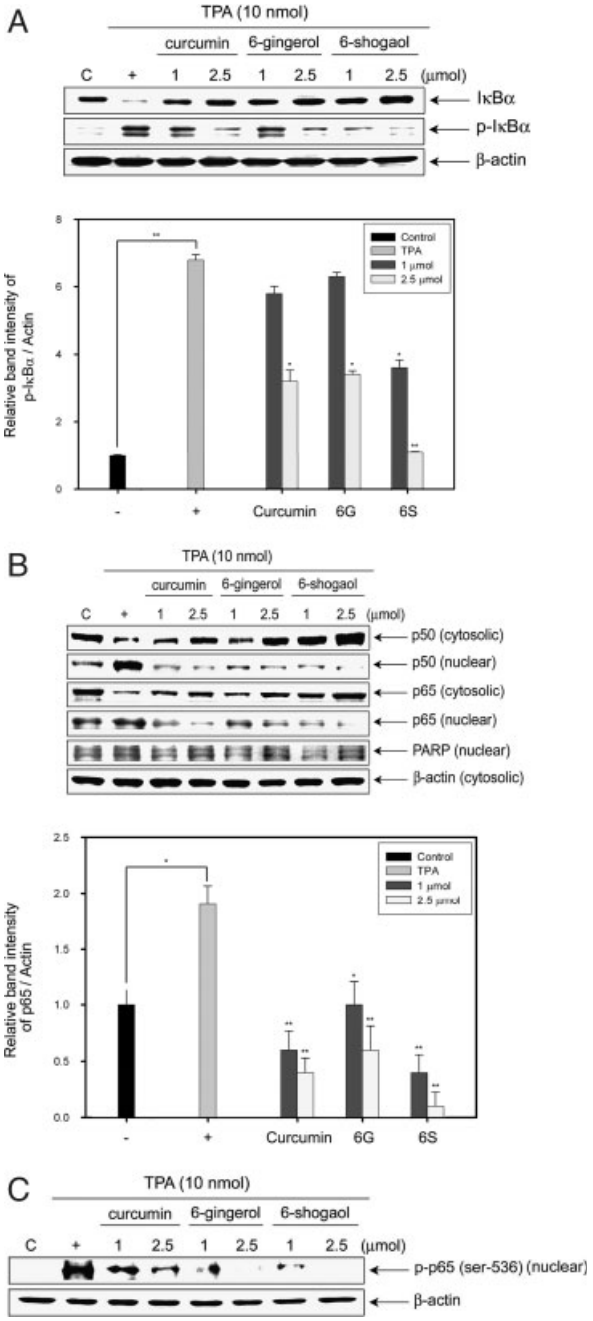

Figure 4. Effect of curcumin, 6-gingerol, and 6-shogaol on phorbol ester-induced NFκB activation. Mice (6 wk of age, six *per* group) were treated topically with 0.2 mL acetone or curcumin, 6-gingerol, and 6-shogaol (1 and 2.5 μmol) in the same volume of acetone 30 min prior to 10 nmol TPA. All mice were (6 wk of age, six *per* group) sacrificed 1 h after TPA treatment, and nuclear and cytosolic extracts from mouse skin were assayed for (A) p-IκB and IκB and by Western blotting analysis. (B) p50, p65, Poly (ADP-ribose) polymerase (PARP), β-actin (C) p-p65 (Ser 536). All analyses are representative of at least three independent experiments. The values under each lane indicate relative density of the band normalized to β-actin.

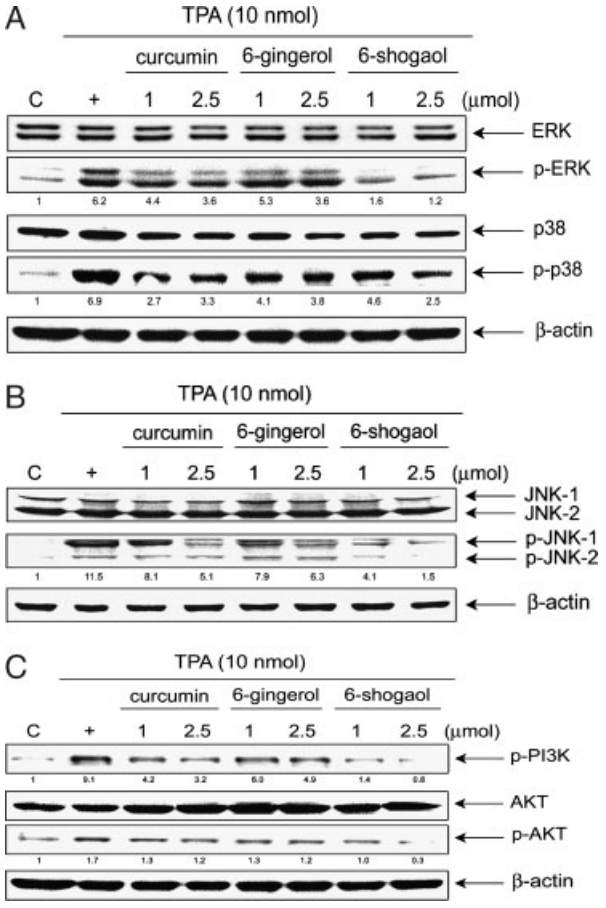

Figure 5. Effects of curcumin, 6-gingerol, and 6-shogaol on TPA-induced activation of ERK, p38 MAPK, JNK, and PI3K/Akt in mouse skin. Mice were treated topically with 0.2 mL acetone or curcumin, 6-gingeriol, and 6-shogaol (1 and 2.5 μmol) in the same volume of acetone 30 min prior to 10 nmol TPA. All mice were sacrificed 1 h after the TPA treatment, and (A) Cell extracts from mouse skin were assayed for ERK, p-ERK, p38, p-p38 with Western blotting analysis. (B) JNK and pJNK. The values under each lane indicate relative density of the band normalized to β-actin. (B) The expression of p-PI3K, Akt, and p-Akt was measured with Western blotting analysis. The values under each lane indicate relative density of the band normalized to β-actin. Data are representative of at least independent experiments, which showed a similar result.

4 Discussion

The results of the present study demonstrate that topical application of 6-shogaol strongly inhibits TPA-stimulated iNOS and COX-2 messenger RNA and protein expression in dose-dependent manner (Figs. 2 and 3) and promotion of skin tumors in mice (Fig. 6). Targeted inhibition of COX-2 without affecting the housekeeping enzyme COX-1 expression is recognized as one of the most promising strategies for chemoprevention of tumor formation. Recently, a third isoform, COX-3, has also reported [27], further research is still required in order to elucidate its precise roles in tumorigenesis. Several lines of evidence has accumulated suggesting that inducible form of COX-2, a central enzyme in the prostaglandin (PG) biosynthetic pathway, plays an important role in tumorigenesis [28]. Knockout and various transgenic approaches and pharmacologic studies show strong evidence for a cause-and-effect relationship between the aberrant COX-2 expression and tumor formation in numerous animal models, including in mouse skin [29]. In a multistage skin carcinogenesis experiment with a classical initiation/promotion using DMBA and TPA, COX-2 deficiency led to a gene dosage-dependent reduction of the papilloma yield and incidence compared with wild-type mice [30]. Moreover, transgenic mice engineered to overexpress COX-2 and elevated PGE_2 levels, treated with DMBA were sufficient to cause tumor development [31, 32]. In contrast, in another study, COX-2 transgenic mice were found to be more resistant, rather than more sensitive, to the development of skin tumor induced by a classical initiation/promotion using DMBA and TPA [33]. Thus, although much of data suggest an important role of COX-2/PGE_2 signaling in the promotion of skin tumorigenesis, its precise role in specific cellular and experimental contexts remains to be clarified. It has been documented that topical application TPA to mouse skin induced epidermal hyperplasia and the epidermis comprise of 95% keratinocytes [34, 35]. We reported previously that the mouse skin tumors that initiated by DMBA and promoted by TPA for 20 wk, markedly increased iNOS and COX-2 levels in skin tumors [36]. Therefore, in the present study, the cell type responded to TPA and the inhibitory agent 6-shogaol could be the same. The related compounds curcumin and 6-gingerol are also active as inhibitors of TPA-induced tumor promotion in mouse skin, but these compounds are less effective than 6-shogaol. Recent reports indicated that compounds having α,β-unsaturated ketone moiety contribute to the inhibition of ERK2 activity but not for other MAPKs such as JNK1 and p38 MAPK [37]. Previous studies suggested that α,β-unsaturated carbonyls are very susceptible to nucleophilic addition reactions with thiols such as glutathione; the most abundant nonprotein thiol *in vivo* [38]. Moreover, NFκB activation could be inhibited by the α,β-unsaturated carbonyl group [39]. IKK consists of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit IKKγ/NEMO. It is suggested that IKKα exhibits a catalytic activity of p65 phosphorylation (Ser536), whereas IKKβ is largely responsible for phosphorylation of both IκBα (Ser32 and p65) [40]. Previous studies indicated that the anti-inflammatory agents aspirin and sodium salicylate specifically inhibit IKKβ activity *in vitro* and *in vivo* through conjugation to IKKβ to reduce ATP binding [41]. We suggested that the α,β-unsaturated carbonyl group in 6-shogaol and curcumin, but not in 6-gingerol, may be responsible for the potent depletory of these thiols in these protein kinase [42]. However, compared with curcumin, 6-shogaol appeared to be smaller and lipophilic and could penetrate more easily into cells and disrupt TPA-induced multiple signaling pathways. Moreover, it is

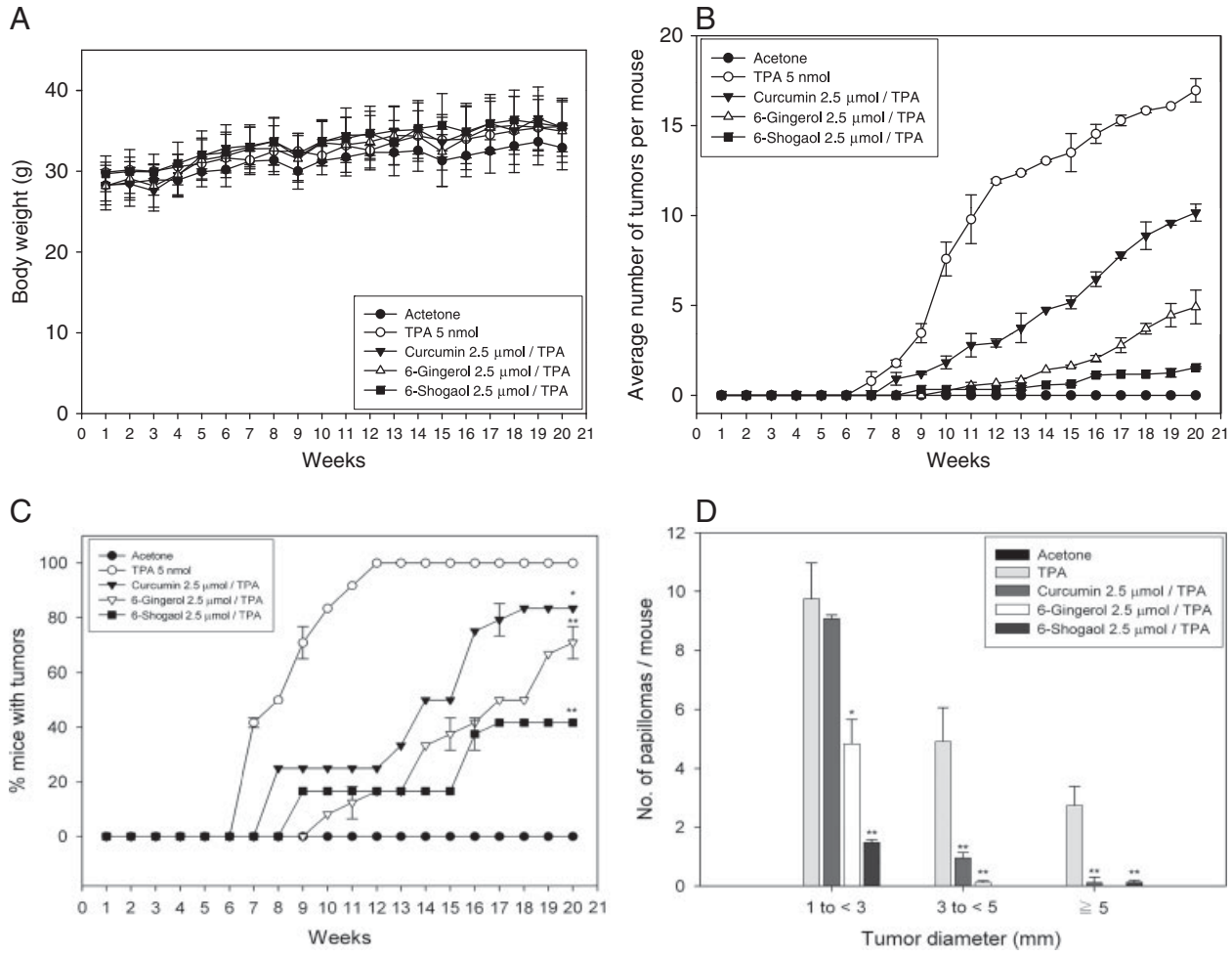

Figure 6. Effects of curcumin, 6-gingerol, and 6-shogaol on DMBA/TPA-induced skin tumorigenesis in ICR mice. Tumor promotion in all mice was initiated with DMBA (200 nmol) and promoted with TPA (5 nmol) twice weekly, starting 1 wk after initiation. Curcumin, 6-gingerol, and 6-shogaol (2.5 μmol) was dissolved in 0.2 mL acetone and topically applied 30 min prior to each TPA treatment. Tumors of at least 1 mm in diameter were counted and recorded weekly, as described in Section 2. (A) The body weight of mice during skin tumor promotion. (B) Average number of tumors *per* mouse (tumor multiplicity). (C) Percentage of tumor-bearing mice (tumor incidence). (D) During the experiment of tumor promotion, the diameters of skin tumors were measured by an electronic digital caliper twice every week. The tumor size was recorded as the average of length × width (millimeter square) *per* mouse. *Significantly different from the corresponding TPA value at $^{*}p<0.05$; $^{**}p<0.01$; indicate statistically significant differences from the TPA-treated group. Statistical analysis was done by Student's *t*-test.

one possibility of observed difference in treatments, pre-treatment of mouse skin with lipophilic compounds could interfere with the absorption of TPA, and we cannot exclude this possibility at this moment. This issue is worth to study further. In this study, we found that 6-shogaol was a stronger inhibitor of iNOS and COX-2 expression in TPA-induced inflammation and tumor promotion than that of curcumin and 6-gingerol. Therefore, we suggested that the α,β-unsaturated carbonyl group in 6-shogaol might influence the conformation of the molecule and modulate its inhibitory effect and could provide new information for the design of anti-inflammatory agents and the study of these functional groups in the future.

Activation of NFκB and AP-1 is necessary for TPA induction of the iNOS and COX-2 [43]. In our study, 6-shogaol was found to inhibit TPA-induced nuclear translocation of NFκB by suppressing phosphorylation of IκBα and p65 and subsequent nuclear translocation of p50 and p65/RelA subunits of NFκB (Fig. 4). In fact, activation of NFκB has been shown to mediate the enhanced expression of inflammation-related genes including iNOS and COX-2 [44].

Both NFκB and PI3K/Akt signaling pathways have emerged as promising molecular targets in the prevention of cancers. Many signaling pathways, including PI3K/Akt, MAPK, and protein kinase C (PKC), have been proposed to respond to TPA stimulation [45]. 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 the inflammatory reaction [46]. Since 6-shogaol can significantly inhibit the induction of iNOS and COX genes and proteins, we have investigated whether 6-shogaol exerts any influence 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, ERK, JNK, and PI3K/Akt. Topical application of 6-shogaol prior to TPA application to mouse skin resulted in the reduction of TPA-induced phosphorylation of p38, ERK MAPK, JNK, and PI3K/Akt in mouse skin (Fig. 5). Since the structure of 6-shogaol is relatively non-polar, it could be either lodged in membrane and affect keratinocyte access to TPA uptake or directly inhibit membrane-bound protein kinase. We do not rule out a possible mechanism in which lipophilic 6-shogaol penetrates cells and probably by competing with the coenzymes or ATP for inhibiting the phosphorylation of MAPKs and PI3K/Akt.

Since PKC is believed to be involved in the regulation of TPA-stimulated inflammation and tumor promotion through the activation of one or more PKC isoforms, additional studies are also needed to determine the effects on curcumin, 6-gingerol, and 6-shogaol on PKC in the presence and absence of TPA.

As predicted by the suppressive efficacies of biochemical markers related to inflammation [36], topical application of 6-shogaol at doses of 2.5 μmol, before TPA treatment during the tumor promotion process, significantly lowered the number and size of papillomas. The probable mechanism of 6-shogaol action is that it down-regulates inflammatory iNOS and COX-2 gene expression in mouse skin by inhibiting the activation of NFκB and by interfering with the activation of PI3K/Akt and MAPKs. Since, papilloma are converted into squamous cell carcinomas, the decrease in the number of papilloma could due to apoptosis [47] in 6-shogaol-treated mice indicates its anticarcinogenic property. 6-Shogaol seems to act as a modulating agent in multiple signaling pathways, thus making it an excellent and novel example of an ideal chemopreventive agent. Further thorough evaluation of 6-shogaol is needed to verify this proposed mechanism.

Interestingly in the present study, 6-shogaol showed markedly more strong anti-tumorigenic effect than that of curcumin and 6-gingerol (Fig. 6). Based on our findings, we suggest that 6-shogaol promotes a strong protective effect against TPA-mediated epithelial carcinogenesis *via* down-regulation of inflammation involving PI3K/Akt/IKK, MAPKs signaling pathways, and NFκB transcription factors, as well as iNOS and COX-2. Above all, this is the first investigation with evidence that 6-shogaol has great potential as a novel chemopreventive agent to be used in the treatment of inflammation associated with tumorigenesis, especially in the prevention and treatment of epithelial skin cancer.

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5 References

[1] Manju, V., Nalini, N., Chemopreventive efficacy of ginger, a naturally occurring anticarcinogen during the initiation, post-initiation stages of 1,2 dimethylhydrazine-induced colon cancer. *Clin. Chim. Acta* 2005, 358, 60–67.

[2] Lantz, R. C., Chen, G.J ., Sarihan, M., Solyom, A. M. *et al.*, The effect of extracts from ginger rhizome on inflammatory mediator production. *Phytomedicine* 2007, 14, 123–128.

[3] Kikuzaki, H., *Ginger for Drug and Spice Purposes*, Technomic Publishing Co., Inc., Pennsylvania, USA 2000, pp. 75–105.

[4] Ravindran, P. N., Babu, K. N., *Ginger: The Genus Zingiber*, CRC Press, Boca Raton 2005, pp. 1, 7–15, 115.

[5] Govindarajan, V. S. Ginger–chemistry, technology, and quality evaluation: part 2. *Crit. Rev. Food Sci. Nutr.* 1982, 17, 189–258.

[6] Govindarajan, V. S. Ginger-chemistry, technology, and quality evaluation: part 1. *Crit. Rev. Food Sci. Nutr.* 1982, 17, 1–96.

[7] Park, K. K., Chun, K. S., Lee, J. M., Lee, S. S., Surh, Y. J., Inhibitory effects of [6]-gingerol, a major pungent principle of ginger, on phorbol ester-induced inflammation, epidermal ornithine decarboxylase activity and skin tumor promotion in ICR mice. *Cancer Lett.* 1998, 129, 139–144.

[8] Balkwill, F., Coussens, L. M., Cancer: an inflammatory link. *Nature* 2004, 431, 405–406.

[9] Pan, M. H., Lai, C. S., Dushenkov, S., Ho, C. T., Modulation of inflammatory genes by natural dietary bioactive compounds. *J. Agric. Food Chem.* 2009, 57, 4467–4477.

[10] Seo, H. J., Park, K. K., Han, S. S., Chung, W. Y. *et al.*, Inhibitory effects of the standardized extract (DA-9601) of Artemisia asiatica Nakai on phorbol ester-induced ornithine decarboxylase activity, papilloma formation, cyclooxygenase-2 expression, inducible nitric oxide synthase expression and nuclear transcription factor kappa B activation in mouse skin. *Int. J. Cancer* 2002, 100, 456–462.

[11] Chun, K. S., Cha, H. H., Shin, J. W., Na, H. K. *et al.*, Nitric oxide induces expression of cyclooxygenase-2 in mouse skin through activation of NF-kappaB. *Carcinogenesis* 2004, 25, 445–454.

[12] Baldwin Jr, A. S., The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu. Rev. Immunol.* 1996, 14, 649–683.

[13] Chio, C. C., Chang, Y. H., Hsu, Y. W., Chi, K. H., Lin, W. W., PKA-dependent activation of PKC, p38 MAPK and IKK in macrophage: implication in the induction of inducible nitric oxide synthase and interleukin-6 by dibutyryl cAMP. *Cell Signal.* 2004, 16, 565–575.

[14] Kim, J. M., Oh, Y. K., Lee, J. H., Im, D. Y. *et al.*, Induction of proinflammatory mediators requires activation of the TRAF, NIK, IKK and NF-kappaB signal transduction pathway in astrocytes infected with Escherichia coli. *Clin. Exp. Immunol.* 2005, 140, 450–460.

[15] Chun, K. S., Kim, S. H., Song, Y. S., Surh, Y. J., Celecoxib inhibits phorbol ester-induced expression of COX-2 and activation of AP-1 and p38 MAP kinase in mouse skin. *Carcinogenesis* 2004, 25, 713–722.

[16] Pan, M. H., Hsieh, M. C., Hsu, P. C., Ho, S. Y. *et al.*, 6-Shogaol suppressed lipopolysaccharide-induced up-expression of iNOS and COX-2 in murine macrophages. *Mol. Nutr. Food Res.* 2008, 52, 1467–1477.

[17] Wood, L. C., Jackson, S. M., Elias, P. M., Grunfeld, C., Feingold, K. R., Cutaneous barrier perturbation stimulates cytokine production in the epidermis of mice. *J. Clin. Invest.* 1992, 90, 482–487.

[18] Grubauer, G., Feingold, K. R., Elias, P. M., Relationship of epidermal lipogenesis to cutaneous barrier function. *J. Lipid Res.* 1987, 28, 746–752.

[19] Chung, W. Y., Park, J. H., Kim, M. J., Kim, H. O. *et al.*, Xanthorrhizol inhibits 12-O-tetradecanoylphorbol-13-acetate-induced acute inflammation and two-stage mouse skin carcinogenesis by blocking the expression of ornithine decarboxylase, cyclooxygenase-2 and inducible nitric oxide synthase through mitogen-activated protein kinases and/or the nuclear factor-kappa B. *Carcinogenesis* 2007, 28, 1224–1231.

[20] Lai, C. S., Li, S., Chai, C. Y., Lo, C. Y. *et al.*, Inhibitory effect of citrus 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone on 12-O-tetradecanoylphorbol 13-acetate-induced skin inflammation and tumor promotion in mice. *Carcinogenesis* 2007, 28, 2581–2588.

[21] Romashkova, J. A., Makarov, S. S., NF-kappaB is a target of AKT in anti-apoptotic PDGF signalling. *Nature* 1999, 401, 86–90.

[22] Chun, K. S., Cha, H. H., Shin, J. W., Na, H. K. *et al.*, Nitric oxide induces expression of cyclooxygenase-2 in mouse skin through activation of NF-kappaB. *Carcinogenesis* 2004, 25, 445–454.

[23] Israel, A., A role for phosphorylation and degradation in the control of NF-kappa B activity. *Trends Genet.* 1995, 11, 203–205.

[24] Chun, K. S., Kim, S. H., Song, Y. S., Surh, Y. J., Celecoxib inhibits phorbol ester-induced expression of COX-2 and activation of AP-1 and p38 MAP kinase in mouse skin. *Carcinogenesis* 2004, 25, 713–722.

[25] Saleem, M., Afaq, F., Adhami, V. M., Mukhtar, H., Lupeol modulates NF-kappaB and PI3K/Akt pathways and inhibits skin cancer in CD-1 mice. *Oncogene* 2004, 23, 5203–5214.

[26] Chan, K. S., Carbajal, S., Kiguchi, K., Clifford, J. *et al.*, Epidermal growth factor receptor-mediated activation of Stat3 during multistage skin carcinogenesis. *Cancer Res.* 2004, 64, 2382–2389.

[27] Chandrasekharan, N. V., Dai, H., Roos, K. L., Evanson, N. K. *et al.*, COX-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drugs: cloning, structure, and expression. *Proc. Natl. Acad. Sci. USA* 2002, 99, 13926–13931.

[28] Greenhough, A., Smartt, H. J., Moore, A. E., Roberts, H. R. *et al.*, The COX-2/PGE2 pathway: key roles in the hallmarks of cancer and adaptation to the tumour microenvironment. *Carcinogenesis* 2009, 30, 377–386.

[29] Muller-Decker, K., Furstenberger, G., Neumann, M., Schnolzer, M., Differential protein expression in the epidermis of wild-type and COX-2 transgenic mice. *Skin Pharmacol. Physiol.* 2006, 19, 89–94.

[30] Tiano, H. F., Loftin, C. D., Akunda, J., Lee, C. A. *et al.*, Deficiency of either cyclooxygenase (COX)-1 or COX-2 alters epidermal differentiation and reduces mouse skin tumorigenesis. *Cancer Res.* 2002, 62, 3395–3401.

[31] Muller-Decker, K., Neufang, G., Berger, I., Neumann, M., Marks, F., Furstenberger, G., Transgenic cyclooxygenase-2 overexpression sensitizes mouse skin for carcinogenesis. *Proc. Natl. Acad. Sci. USA* 2002, 99, 12483–12488.

[32] Rundhaug, J. E., Pavone, A., Kim, E., Fischer, S. M., The effect of cyclooxygenase-2 overexpression on skin carcinogenesis is context dependent. *Mol. Carcinog.* 2007, 46, 981–992.

[33] Bol, D. K., Rowley, R. B., Ho, C. P., Pilz, B. *et al.*, Cyclooxygenase-2 overexpression in the skin of transgenic mice results in suppression of tumor development. *Cancer Res.* 2002, 62, 2516–2521.

[34] Wood, L. C., Jackson, S.M., Elias, P.M., Grunfeld, C. *et al.*, Cutaneous barrier perturbation stimulates cytokine production in the epidermis of mice. *J. Clin. Invest.* 1992, 90, 482–487.

[35] Pedranzini, L., Leitch, A., Bromberg, J., Stat3 is required for the development of skin cancer. *J. Clin. Invest.* 2004, 114, 619–622.

[36] Lai, C. S., Li,S., Chai, C. Y., Lo, C. Y. *et al.*, Anti-inflammatory and antitumor promotional effects of a novel urinary metabolite, 3′,4′-didemethylnobiletin, derived from nobiletin. *Carcinogenesis* 2008, 29, 2415–2424.

[37] Ohori, M., Kinoshita, T., Yoshimura, S., Warizaya, M. *et al.*, Role of a cysteine residue in the active site of ERK and the MAPKK family. *Biochem. Biophys. Res. Commun.* 2007, 353, 633–637.

[38] Boyland, E., Chasseaud, L. F., Enzymes catalysing conjugations of glutathione with alpha-beta-unsaturated carbonyl compounds. *Biochem. J.* 1968, 109, 651–661.

[39] Van, L. C., Van, C. M., Lee, J. J., Jung, S. H., Exploration of essential structure of malloapelta B for the inhibitory activity against TNF induced NF-kappaB activation. *Arch. Pharm. Res.* 2006, 29, 840–844.

[40] Sizemore, N., Lerner, N., Dombrowski, N., Sakurai, H., Stark, G. R., Distinct roles of the Ikappa B kinase alpha and beta subunits in liberating nuclear factor kappa B (NF-kappa B) from Ikappa B and in phosphorylating the p65 subunit of NF-kappa B. *J. Biol. Chem.* 2002, 277, 3863–3869.

[41] Yin, M. J., Yamamoto, Y., Gaynor, R. B., The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta. *Nature* 1998, 396, 77–80.

[42] Surh, Y. J., Lee, S. S., Enzymatic reduction of shogaol: a novel biotransformation pathway for the alpha,beta-unsaturated ketone system. *Biochem. Int.* 1992, 27, 179–187.

[43] Saleem, M., Afaq, F., Adhami, V. M., Mukhtar, H., Lupeol modulates NFskappaB and PI3K/Akt pathways and inhibits skin cancer in CD-1 mice. *Oncogene* 2004, 23, 5203-5214.

[44] Sun, X. F., Zhang, H., NFKB and NFKBI polymorphisms in relation to susceptibility of tumour and other diseases. *Histol. Histopathol.* 2007, 22, 1387-1398.

[45] Murayama, K., Kimura, T., Tarutani, M., Tomooka, M. *et al.*, Akt activation induces epidermal hyperplasia and proliferation of epidermal progenitors. *Oncogene* 2007, 26, 4882-4888.

[46] Carpenter, C. L., Cantley, L. C., Phosphoinositide kinases. *Curr. Opin. Cell Biol.* 1996, 8, 153–158.

[47] Pan, M. H., Hsieh, M. C., Kuo, J. M., Lai, C. S. *et al.*, 6-Shogaol induces apoptosis in human colorectal carcinoma cells via ROS production, caspase activation, and GADD 153 expression. *Mol. Nutr. Food Res.* 2008, 52, 527-537.

