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PAPER

Pterostilbene, a natural analogue of resveratrol, potently inhibits 7,12-dimethylbenz[*a*]anthracene (DMBA)/12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced mouse skin carcinogenesis

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We reported previously that pterostilbene, a natural analogue of resveratrol from blueberries, strongly suppressed lipopolysaccharide-induced up-expression of inducible NO synthase (iNOS) and cyclooxygenase-2 (COX-2) in murine macrophages. In this study, we further investigated pterostilbene's molecular mechanism of action and its anti-tumor properties. Pretreatment with pterostilbene has resulted in the reduction of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced nuclear translocation of the nuclear factor-κB (NFκB) subunits. Pterostilbene also reduced TPA-induced phosphorylation of IκBα and p65 and caused subsequent degradation of IκBα. Moreover, pterostilbene markedly suppressed TPA-induced activation of extracellular signal-regulated kinase (ERK)1/2, p38 mitogen-activated protein kinase (MAPK), c-Jun N-terminal kinase (JNK)1/2, phosphatidylinositol 3-kinase (PI3K) and Akt, which are upstream of NFκB and activator protein 1 (AP-1). Furthermore, pterostilbene significantly inhibited 7,12-dimethylbenz[*a*]anthracene (DMBA)/TPA-induced skin tumor formation measured by the tumor multiplicity of papillomas at 20 weeks. The presented data has, for the first time, revealed that pterostilbene is an effective anti-tumor agent that functions by downregulating inflammatory iNOS and COX-2 gene expression in mouse skin. It is suggested that pterostilbene is a novel functional agent capable of preventing inflammation-associated tumorigenesis.

1. Introduction

Pterostilbene (*trans*-3,5-dimethoxy-4′-hydroxystilbene), a natural dimethylated analogue of resveratrol from blueberries, is known to have diverse pharmacological activities.[1] Recent studies have exhibited that pterostilbene and resveratrol possess anti-inflammatory, antioxidant and anticarcinogenic properties which may be responsible for their cancer chemopreventive potency.[2,3] Our previous studies reported that dietary administration of pterostilbene against the formation of azoxymethane (AOM)-induced colonic aberrant crypt foci (ACF) preneoplastic lesions and adenomas in male Institute of Cancer Research (ICR) mice.[4] We previously reported that pterostilbene promotes a strong protective effect against TPA-mediated metastasis *via* down-regulation of early and long-term inside-out signaling processes.[5] It is well-established that inflammation is causally linked to carcinogenesis and acts as a driving force in pre-malignant and malignant transformations of cells.[6,7] Topical application of TPA to mice leads to edema and promotes DMBA-initiated mouse papilloma formation by enhancing iNOS, COX-2 and ornithine decarboxylase (ODC) protein expression.[8] Specific iNOS and COX-2 inhibitors are able to counteract these biological events.[9] Activated NFκB and AP-1 often facilitate the transcription of numerous genes, including iNOS and COX-2, resulting in inflammation and tumorigenesis. TPA activates NF-κB activation through inhibitor κB (IκB) kinase (IKK)-mediated phosphorylation-induced proteasomal degradation of the IκB.The subsequent ubiquitination and proteasomal degradation of IκB frees NFκB to translocate to the nucleus.[10] These kinases can be activated through phosphorylation by upstream kinases, including NFκB-inducing kinase and mitogen-activated protein kinase.[11,12] 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 PI3K/Akt pathways.[13]

A recent study showed that topically applied pterostilbene is equally potent as resveratrol in inhibiting TPA activated NFκB, AP-1, COX-2 and iNOS in mouse epidermis.[14] However, the antitumor-promoting effects of pterostilbene and resveratrol on DMBA/TPA-induced skin tumorigenesis in mice are still undefined. Thus, the present study was designed to investigate

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whether pterostilbene suppresses the proliferation activity of mouse skin following TPA application and investigate the inhibitory effect of pterostilbene on the DMBA-initiated, TPA-promoted two-stage skin carcinogenesis model, including tumor incidence, multiplicity and volume. We have also evaluated the effects of the related compound resveratrol as a potential inhibitor of tumor promotion and its molecular mechanism of action.

2. Materials and methods

2.1. Chemicals

Pterostilbene and resveratrol were gifts from Sabinsa Corp. (East Windsor, NJ). The purity of pterostilbene and resveratrol was determined by high-performance liquid chromatography (HPLC) as higher than 99.2%. 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.

2.2. Animals

All animal experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee of the National Kaohsiung Marine University (IACUC, NKMU, policy agreement #099-AAA9-02). Procedures were realised according to Taiwan law on care and use of laboratory animals. Female Institute of Cancer Research (ICR) mice at 5–6 weeks old were obtained from the BioLASCO Experimental Animal Center (Taiwan Co., Ltd, BioLASCO, Taipei, Taiwan). 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 compounds. Pterostilbene, resveratrol 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 pterostilbene or resveratrol in 200 μL of acetone,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 the 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.[15,16] 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 ethylene glycol-bis(aminoethylether)-tetraacetic acid (EGTA), 1 mM phenylmethanesulfonyl fluoride, 1% NP-40 and 10 μg mL^{-1} 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% sodium dodecyl sulfate (SDS), 25 mM ethylenediaminetetraacetic acid (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 μm immobile membrane (polyvinylidene difluoride; Millipore Corp., Bedford, MA) 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), COX-2 monoclonal antibodies (Transduction Laboratories, BD Biosciences, Lexington, KY), phospho-p65 (Ser536), phospho-p38 (Thr180/Tyr182), phospho-ERK1/2 (Thr202/Tyr204), ERK, JNK and p38 polyclonal antibodies (Cell Signaling Technology, Beverly, MA), phospho-IκBα (Ser32/Ser36), phospho-Akt (Ser473), Akt polyclonal antibodies (Upstate Biotechnology, Inc., Lake Placid, NY). 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, Alpha Innotech Corp., San. Leandro, CA). All the membranes were stripped and re-probed for β-actin (Sigma Chemical Co., Saint Louis, MO) as the loading control.

2.4. Semiquantitative reverse-transcription polymerase chain reaction (RT-PCR)

Total RNA from the epidermal skin was extracted using TRIZOL reagent according to the supplier's protocol. The RNA concentration was determined by measuring the ultraviolet absorbance at 260 and 280 nm, and the RNA was stored at −70 °C until PCR analysis. Changes in the steady-state concentration of mRNA in iNOS, COX-2 and β-actin were assessed by RT-PCR. A total of 2 μg RNA was transcribed into cDNA using SuperScript II Reverse Transcriptase (Invitrogen, Renfrewshire, U.K.) in a final volume of 20 μL. RT reactions were performed at 50 °C for 50 min and 70 °C for 15 min in a Gene Cycler thermal cycler (Bio-Rad). The thermal cycle conditions were initiated at 95 °C for 1 min and 30 cycles of amplification (94 °C for 30 s, 58 °C for 25 s and 72 °C for 1 min), followed by extension at 72 °C for 3 min. The PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. Amplification of β-actin served as a control for sample loading and integrity. PCR was performed on the cDNA using the following sense and antisense primer: iNOS, forward primer 5′-CCCTTCCGAAGTTTCTGGCAG CAGC-3′ (2944–2968), reverse primer 5′-GGCTGTCAGAGAG CCTCGTGGCTTTGG-3′ (3416–3440); COX-2, forward primer 5′-GGAGAGACTATCAAGATAGTGATC-3′ (1094–1117),

reverse primer 5′-ATGGTCAGTAGACTTTTACAGCTC-3′ (1931–1954); β-actin, forward primer 5′-ACCAACTGGGAC GATATGGAGAAGA-3′, reverse primer 5′-TACGACCA GAGGCATACAGGGACAA-3′. Confirmation of the correct amplicons was obtained by direct DNA sequencing of the PCR products.

2.5. Preparation of cytosolic and nuclear extracts from mouse skin

Cytosolic and nuclear protein extraction was performed as previously described.[17] 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% Nonident P-40 (NP-40), vortexed and centrifuged for 2 min at 15 994 × *g*. 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 minutes and then centrifuged at 15 994 × *g* for 5 minutes. 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. Proliferating cell nuclear antigen (PCNA) immunohistochemistry

For indirect PCNA immunochemistry, the deparaffinized skin sections (4 μm) were incubated with 1.2% H_2O_2 in phosphate buffered saline (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 and then applied to each section overnight at 4 °C. After washing with PBS, the sections were incubated with a biotin-conjugated horseradish peroxidase secondary antibody (1 : 200; Vector Laboratories, Burlingame, CA, USA) for 1 h at room temperature. Finally, the peroxidase was detected using the 3,3-diaminobenzidine tetrahydrochloride reaction, which produced a brown label in the epidermis. The number of PCNA-positive staining cells was counted in six different fields (×200) at both ends as well as in the middle for each section. The PCNA index was counted at six different areas of each section using the image analysis system and was expressed as the number of positive squamous cells divided by the total number of squamous cells × 100.

2.7. Two-stage tumorigenesis in mouse skin

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. The anti-tumor promoting activity of pterostilbene or resveratrol was examined by standard initiation-promotion with DMBA and TPA, as reported previously.[18] Female ICR mice were randomly divided into five groups, each consisting of 12 animals. 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 weeks. For the other groups, the mice were treated with pterostilbene (1 and 5 μmol in 200 μL acetone) or resveratrol (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.

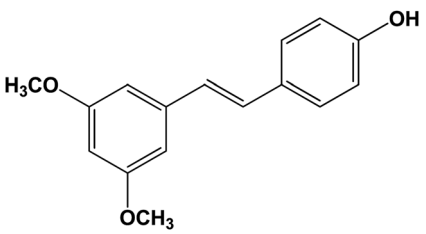
2.8. Statistical analysis

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 pterostilbene or resveratrol plus TPA-treated groups. A P value < 0.05 was considered statistically significant.

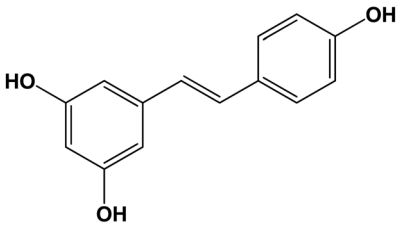
3. Results

3.1. Inhibitory effects of pterostilbene and resveratrol on TPA-induced iNOS and COX-2 expression in mouse skin

The anti-inflammatory activity of pterostilbene and resveratrol (Fig. 1) can be demonstrated by its effect on iNOS and COX-2 expression in TPA-stimulated mouse skin. We have shown



Pterostilbene (PSB)



Resveratrol (R)

Fig. 1 Chemical structures of pterostilbene (PSB) and resveratrol (R).

previously that TPA, a mitogen and well-known promoter of skin tumorigenesis, stimulates iNOS and COX-2 expression in mouse skin.[19] In our previous study, 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 h and 4 h, respectively.[20] As shown in Fig. 2, topical application of pterostilbene 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, resveratrol (5 μmol) strongly suppressed the expression of COX-2 proteins. To investigate whether or not pterostilbene and resveratrol has any influence on TPA-induced *inos* and *cox-2* gene expression, we applied pterostilbene at 1 or 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). Resveratrol also more strongly suppressed *COX-2* gene expression than pterostilbene.

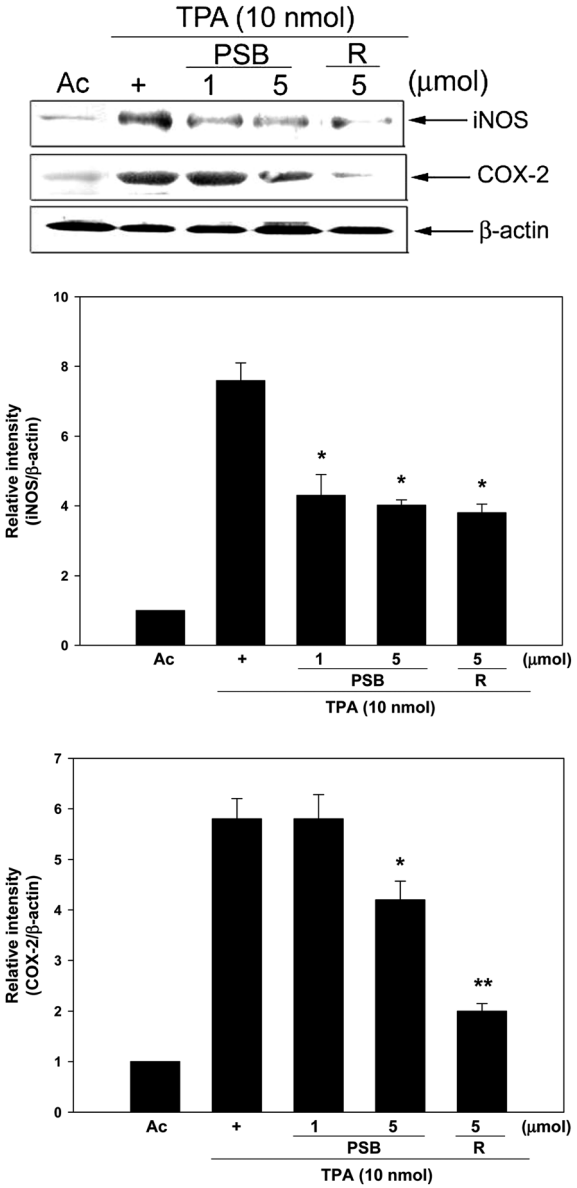


Fig. 2 Inhibitory effects of pterostilbene and resveratrol on TPA-induced iNOS and COX-2 protein expression. Female ICR mice were treated topically with acetone alone or 10 nmol TPA on dorsal skins for indicated time periods. Mice (6-weeks of age, six per group) were treated topically with 0.2 mL acetone or pterostilbene (1 and 5 μmol) and resveratrol (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 protein levels were normalized to β-actin using a densitometer. * $P < 0.05$; ** $P < 0.01$ indicates statistically significant differences from the TPA-treated group. Statistical analysis was done by the Student's *t*-test. PSB, pterostilbene; R, resveratrol.

3.2. Inhibitory effect of pterostilbene and resveratrol on TPA-induced NFκB activation in mouse skin

iNOS and COX-2 are frequently regulated by activating the NFκB signaling pathway,[9,21] and NFκB activation and nuclear translocation are preceded by the phosphorylation and proteolytic degradation of IκBα.[22] Therefore, it is important to investigate how pterostilbene and resveratrol inhibit the 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 pterostilbene and its effect on IκBα degradation. Topical application of TPA led to phosphorylation and degradation of IκBα, which were significantly repressed by pterostilbene pre-treatments (Fig. 4A). Resveratrol has the same effect compared to pterostilbene. Moreover, by the topical application of pterostilbene onto mouse skin (prior to TPA application), we found that TPA-induced NFκB nuclear translocation was inhibited by the pterostilbene pre-treated animal groups in a dose-dependent manner (Fig. 4B). Compared to pterostilbene, resveratrol (5 μmol) has the same activity. In this experiment, poly(ADP-ribose) polymerase (PARP), a nuclear protein and β-actin, a cytosolic protein, were used as controls to confirm that there was no contamination during the extraction of each fraction.

3.3. Inhibitory effect of pterostilbene and resveratrol on TPA-induced activation of MAP kinases, JNK and phosphatidyl inositol kinase (PI3K), Akt/protein kinase B

MAP kinases 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 and AP-1in mouse skin.[23,24] Therefore, we investigated the effects of pterostilbene and resveratrol on TPA-induced phosphorylation of p38 MAPK, JNK, ERK, PI3K and Akt in mouse skin. Western blot analysis revealed that topical application of TPA alone caused a significant increase in the phosphorylation of p38 MAPK, JNK, ERK, PI3K and Akt in mouse skin compared to vehicle-treated controls. Pre-treatment of pterostilbene strongly inhibited the

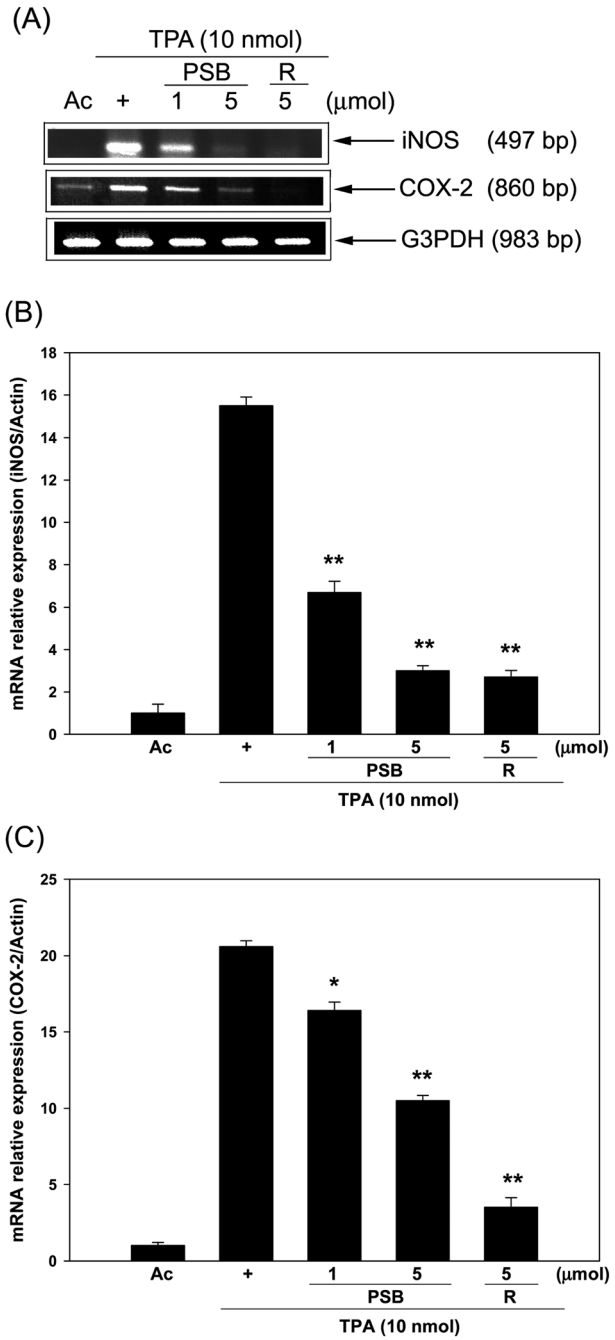


Fig. 3 Effects of pterostilbene and resveratrol on *iNOS* and *COX-2* gene expression on topical application of TPA in mouse skin. Mice (6-weeks of age, six per group) were treated topically with 0.2 mL acetone or pterostilbene (1 and 5 μmol) and resveratrol (5 μmol) in the same volume of acetone 30 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 μg of cDNA were subject to real time PCR. The PCR product was resolved in 1.5% agarose gel. Quantification on of the mRNA levels of the *iNOS* and *COX-2* genes were performed by densitometric analysis of the agarose gel. The data is representative of at least three independent experiments. Data are mean ± SE. * $P < 0.05$; ** $P < 0.01$ were *verse* TPA alone. PSB, pterostilbene; R, resveratrol.

phosphorylation of p38, JNK, ERK and PI3K and Akt in TPA-treated mouse skin in a dose-dependent manner (Fig. 5). We also observed that pre-application of resveratrol prior to TPA treatment attenuated TPA-induced phosphorylation of p38 MAPK, JNK, ERK, PI3K and Akt in mouse skin (Fig. 5). The results of these studies indicate that resveratrol is more active than pterostilbene. Interestingly, pterostilbene more strongly inhibited the phosphorylation of p38 than resveratrol. More importantly, no change was observed in the total epidermal p38, JNK, ERK and Akt content in mice treated with both TPA and pterostilbene compared to vehicle-treated control.

3.4. Effect of pterostilbene and resveratrol on TPA-induced tumor promotion in mouse epidermis

We performed a study on pterostilbene and resveratrol inhibition of TPA-induced cell proliferation. Immunohistochemical analysis of PCNA was used to assess the proliferation activity during tumor promotion. The PCNA-labeling index increased in the epidermis of the TPA-treated mice compared with the control. Pretreatment with 1 and 5 μmol of pterostilbene prior to TPA application significantly reduced the PCNA-labeling index in a dose-dependent manner (Fig. 6). Interestingly, pterostilbene was more effective in inhibiting PCNA positive cells. Since application of 5 μmol of pterostilbene to mouse skin significantly inhibited various molecular targets that play significant roles in the progression of skin tumors, we selected this dose and compared with resveratrol for assessing the anti-tumor promoting potential of pterostilbene in DMBA-initiated mouse skin. As shown in Fig. 7A, at the end of the 20 weeks TPA-induced promotion experiment, no statistically significant difference in body or organ weight was observed between the mice treated with and without pterostilbene or resveratrol, indicating that the topical application of pterostilbene or resveratrol did not cause any toxicity. Mice initiated with DMBA and promoted with TPA twice weekly for 20 weeks developed an average of 38.0 tumors/mouse. When pterostilbene was applied at a dose 1 or 5 μmol to the shaven backs of mice 30 min prior to each TPA application, the average number of papillomas per mouse was reduced by 50.0 and 63.2%, respectively. However, pre-treatment with resveratrol reduced the number of tumors per mouse by 44.7% at a 5 μmol dose (Fig. 7B). Tumor incidence in this positive control group and pterostilbene were 100% 20 weeks after promotion. Pretreatment with 5 μmol of resveratrol 30 min prior to each application of TPA saw that the incidence was reduced by 20 % (Fig. 7C). In contrast, DMBA-initiated dorsal skin followed by repeated application of acetone produced no tumors. The tumor promotion data were analyzed in terms of size distribution of papillomas and were compared with the DMBA-initiated and TPA-promoted mouse skin. The number of papillomas (1 to <3 mm in diameter) and tumor weight per mouse were significantly inhibited in the pterostilbene and resveratrol treated groups (Fig. 7D and E). In addition, pterostilbene (5 μmol) treatment showed a significant inhibition in tumors at sizes of 3 to <5 mm as is evident in a significant reduction in tumor size and tumor weight compared with resveratrol and the DMBA-initiated and TPA-promoted mouse skin. The animals that were started on DMBA and treated twice weekly with pterostilbene and resveratrol were devoid of any skin

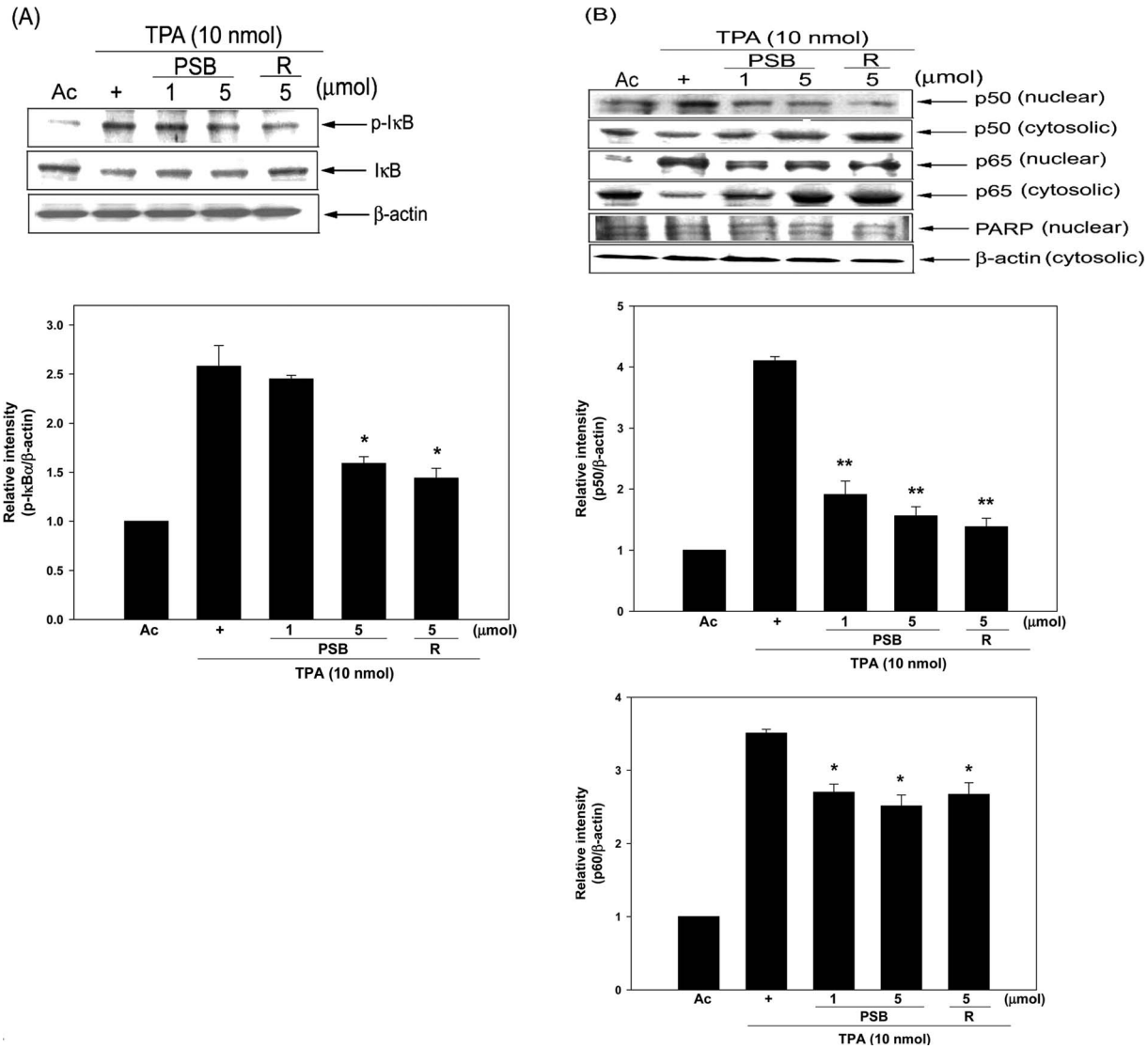


Fig. 4 Effect of pterostilbene and resveratrol on TPA-induced NF-κB activation. Mice (6-weeks of age, six per group) were treated topically with 0.2 mL acetone or pterostilbene (1 and 5 μmol) and resveratrol (5 μmol) in the same volume of acetone 30 min prior to 10 nmol TPA. All mice were (6-weeks of age, six per group) sacrificed 1 h after TPA treatment, and nuclear and cytosolic extracts from the mouse skin were assayed for (A) p-IκBα and IκBα and by western blotting analysis; (B) p50, p65, Poly (ADP-ribose) polymerase (PARP), β-actin. All analyses are representative of at least three independent experiments. The values under each lane indicate the relative density of the band normalized to β-actin. * $P < 0.05$; ** $P < 0.01$ indicates statistically significant differences from the TPA-treated group. Statistical analysis was done by the Student's *t*-test. PSB, pterostilbene; R, resveratrol.

tumors throughout the experiment (data not shown), suggesting that pterostilbene and resveratrol are not tumor promoters. The inhibitory effects of pterostilbene were more potent than those of resveratrol.

4. Discussion

Several epidemiological studies have shown that chronic inflammation predisposes individuals to various types of cancer. Chemoprevention by using natural bioactive compounds is gaining the attention of consumers and researchers as an important approach for management of various cancers.[25] Our previous studies showed that pterostilbene was found to be as effective as resveratrol in preventing AOM-induced colon tumorigenesis *via* activation of the NF-E2-related factor 2 (Nrf2)-mediated antioxidant signaling pathway and in inhibiting tumor invasion.[1,5] Moreover, pterostilbene has been demonstrated to have a cancer chemopreventative activity similar to that of resveratrol, and is cytotoxic to a number of cancer cell lines.[26] Our studies confirmed the earlier observation of Cichocki *et al.*,[14] that topical application of pterostilbene is equally as potent as resveratrol in inhibiting TPA-induced iNOS and COX-2 expression by inhibiting the activation of NFκB *via* blocking the translocation of p65 to the nucleus. In this study, employing a two-stage mouse skin carcinogenesis protocol, we clearly demonstrated that topical application of pterostilbene at doses of 1 and 5 μmol before TPA treatment during the tumor promotion process significantly lower the number and size of papillomas. In

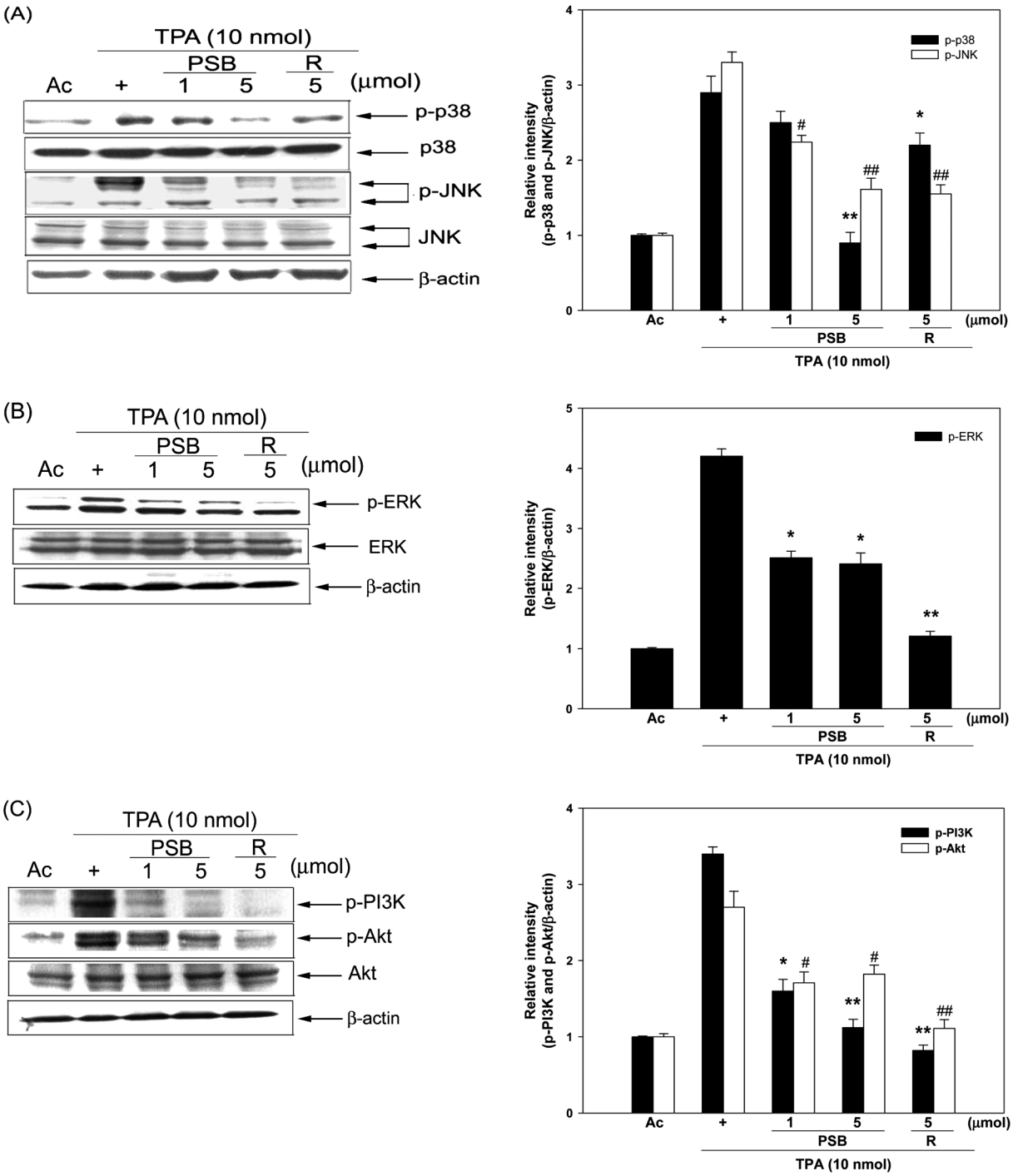


Fig. 5 Effects of pterostilbene and resveratrol on TPA-induced activation of p38 MAPK, JNK, ERK and PI3K/Akt in mouse skin. Mice (6-weeks of age, six per group) were treated topically with 0.2 mL acetone or pterostilbene (1 and 5 μmol) and resveratrol (5 μmol) in the same volume of acetone 30 min prior to 10 nmol TPA. All mice were sacrificed 1 h after the TPA treatment and (A) protein extracts from mouse skin were assayed for p–p38, p38, p-JNK, JNK with western blotting analysis; (B) pERK and ERK. (C) The expression of p-PI3K, p-Akt and Akt was measured with western blotting. The values under each lane indicate relative density of the band normalized to β-actin. Data are representative of at least three independent experiments, which showed a similar result. */# $P < 0.05$; **/## $P < 0.01$ indicates statistically significant differences from the TPA-treated group. Statistical analysis was done by the Student's *t*-test. PSB, pterostilbene; R, resveratrol.

our current study, it is most interesting that we demonstrated that pterostilbene was more potent than resveratrol in inhibiting DMBA/TPA-induced skin tumor formation measured by the tumor multiplicity of papillomas at 20 weeks (Fig. 7). Although resveratrol exerted more effectively than pterostilbene during short term treatment in inhibiting TPA-induced the levels of COX-2 gene and protein (Fig. 2 and 3), its antitumorigenetic activity may be limited by its low bioavailability. Pterostilbene

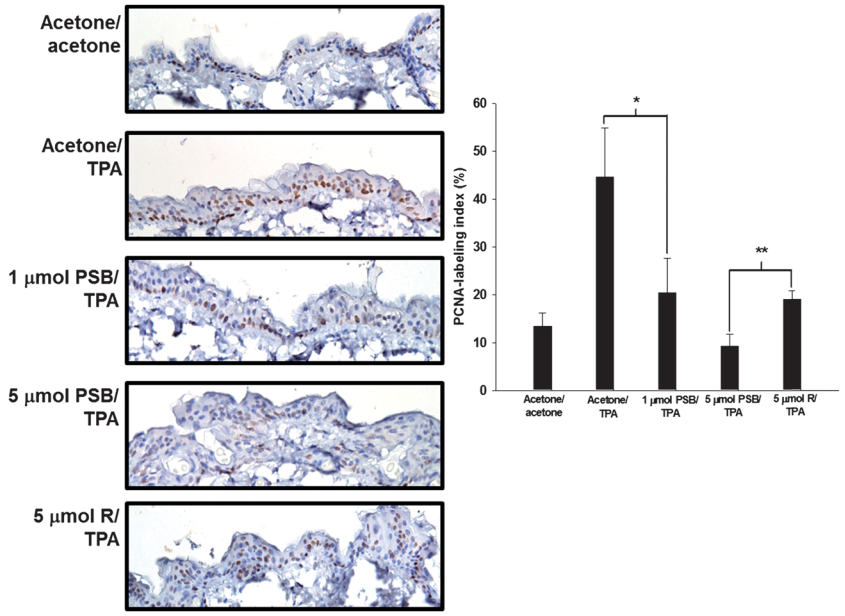


Fig. 6 Inhibitory effects of pterostilbene and resveratrol on TPA-induced PCNA-positive cell in mouse skin. Mice (6-weeks of age, six per group) were treated topically with 0.2 mL acetone or pterostilbene (1 and 5 μmol) and resveratrol (5 μmol) in the same volume of acetone 30 min prior to 10 nmol TPA. All mice were sacrificed 1 h after the TPA treatment. PCNA-labeling index (%) as described in Materials and Methods. Significantly different from the corresponding TPA value at $*P < 0.05$; $** P < 0.01$ (pterostilbene *versus* resveratrol). Statistical analysis was done by the Student's *t*-test. PSB, pterostilbene; R, resveratrol.

may have improved bioavailability because of the substitute of two hydroxyl groups on resveratrol with two methoxy groups, which increase lipophilicity. This effect was also demonstrated in our previous study, that pterostilbene is more potent than resveratrol in preventing AOM-induced colon tumorigenesis.[1] Our present study demonstrated that pterostilbene could significantly inhibit the induction of iNOS and COX genes and proteins. Many signaling pathways, including PI3K/Akt and ERK/MAPK, have been proposed to respond to TPA stimulation.[20,27] We also clearly demonstrated that topical application of TPA resulted in the activation of p38, JNK, ERK, PI3K and Akt. Pterostilbene administered directly to the mouse skin prior to TPA application resulted in the reduction of TPA-induced phosphorylation of p38, JNK, ERK, PI3K and Akt in mouse skin (Fig. 5). Interestingly, pterostilbene decreases p38 phosphorylation to a greater extent compared with resveratrol, which suggests that MAP kinase kinase kinase (MEKK)/MAP kinase kinase (MKK)/p38 pathway is mainly affected by pterostilbene. Since the structure of pterostilbene is relatively non-polar, it could either be lodged in the membrane and affect keratinocyte access to TPA uptake, or directly inhibit membrane-bound protein kinase. We cannot rule out the possibility that pterostilbene with lipophilic properties may penetrate cells and inhibit MAPKs, PI3K and Akt by competition with the coenzymes or ATP. This issue is worth studying further. Since protein kinase C (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,[28] additional studies are also needed to determine the effects on pterostilbene on PKC in the presence and absence of TPA. Here we suggest that pterostilbene suppressed TPA-induced protein levels of iNOS and COX-2 by inactivating NFκB by blocking the activation of upstream p38 MAP kinases, which provides a mechanistic basis of its anti-inflammatory and anti-tumor promoting activity in mouse skin.

In conclusion, the results of our present study suggests that pterostilbene promotes a strong protective effect against TPA-mediated epithelial carcinogenesis. This is the first investigation with evidence that pterostilbene might be a potential candidate for the development of anti-cancer drugs for use in the treatment of inflammation associated with tumorigenesis, especially in the prevention and treatment of epithelial skin cancer.

Conflict of interest

The authors have declared no conflict of interest.

Abbreviations

iNOS	Inducible nitric oxide synthase;
NO	Nitric oxide;
COX-2	Cyclooxygenase-2;
NF-κB	Nuclear factor-κB;
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-regulated kinase;
PCR	Polymerase chain reaction;
PI3K	Phosphatidylinositol 3-kinase.

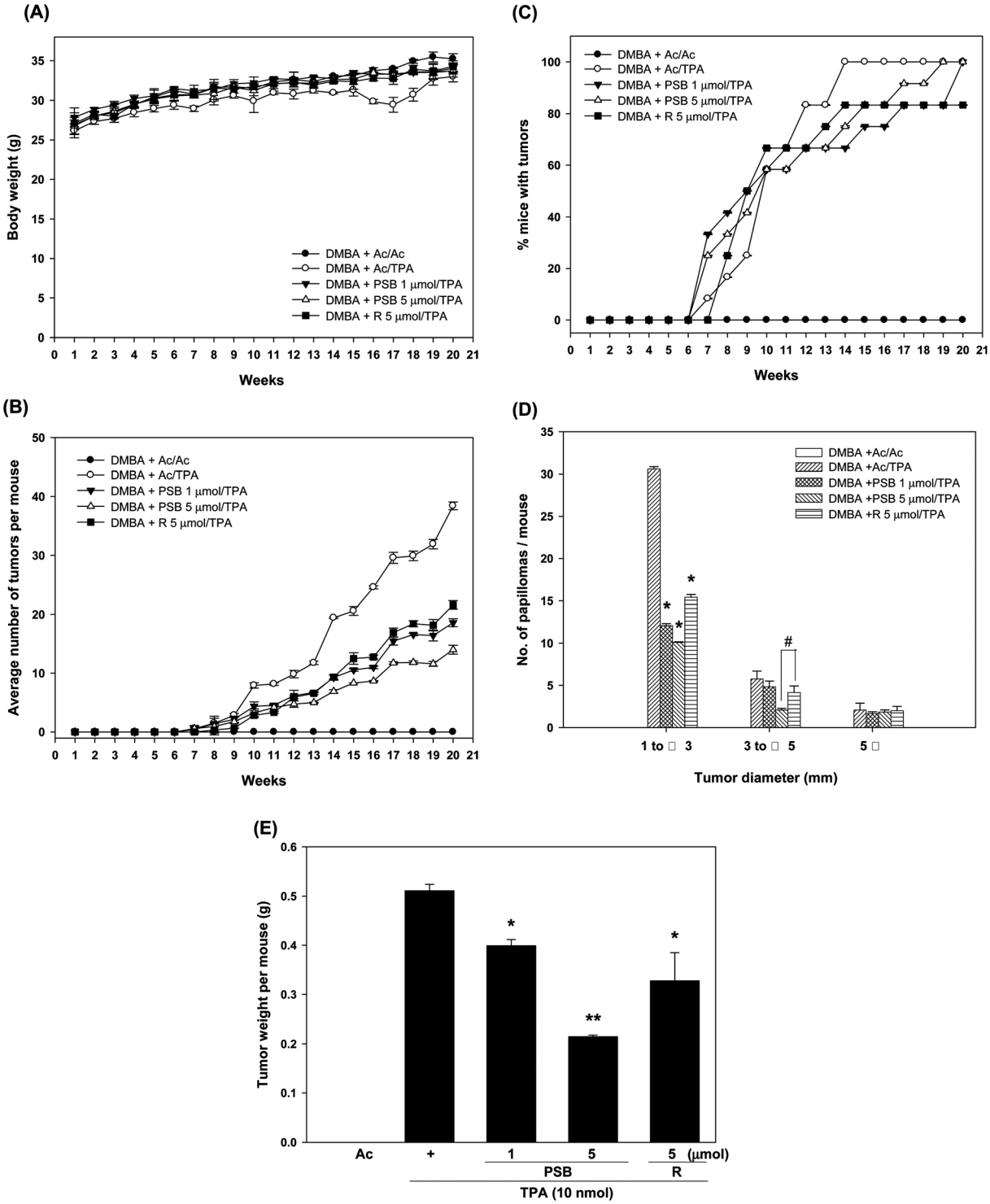


Fig. 7 Antitumor-promoting effects of pterostilbene and resveratrol on DMBA/TPA-induced skin tumors. Tumor promotion in all mice was initiated with DMBA (200 nmol) and promoted with TPA (5 nmol) twice weekly, starting 1 week after initiation. Pterostilbene (1 and 5 μmol) and resveratrol (5 μmol) was 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 the Materials and methods section. (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. (E) Tumor weight per mouse in DMBA and TPA treated mice. * Significantly different from the corresponding TPA value at $P < 0.05$. # Indicates statistically significant differences from resveratrol-treated group at $P < 0.05$. Statistical analysis was done by the Student's *t*-test. PSB, pterostilbene; R, resveratrol.

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