DOI 10.1002/mnfr.200700466 Mol. Nutr. Food Res. 2008, *52*, S62 – S70

Research Article

Pterostilbene is equally potent as resveratrol in inhibiting 12-*O*-tetradecanoylphorbol-13-acetate activated NFκB, AP-1, COX-2, and iNOS in mouse epidermis

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Resveratrol, a phytoalexin present in grapes, has been reported to inhibit multistage mouse skin carcinogenesis. Recent studies showed that topically applied resveratrol significantly inhibited cyclooxygenase-2 (COX-2) expression and activation of nuclear factor-κB (NF-κB) induced by tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in mouse epidermis. The aim of the present study was to further explore the effect of resveratrol on TPA-induced signaling pathways in mouse epidermis and to compare with its dimethylether, pterostilbene. Resveratrol and pterostilbene significantly reduced activator protein 1 (AP-1) and NF-κB activation. In the case of AP-1, the binding of c-Jun subunit was particularly affected, while only slight effect on c-Fos binding to TPA-responsive element (AP-1 binding consensus sequence) (TRE) site was observed. Both stilbenes inhibited the activation of NF-κB by blocking the translocation of p65 to the nucleus and increasing the retention of IκBa in the cytosol. The latter might be related to decreased activity of IκB kinase and/or proteasome 20S. Reduced activation of transcription factors decreased the expression and activity of COX-2 and inducible nitric oxide synthase (iNOS). In most assays, pterostilbene was either equally or significantly more potent than resveratrol. Pterostilbene might show higher biological activity due to its possible better bioavailability, since substitution of hydroxy with methoxy group increases lipophilicity.

Keywords: AP-1 / COX-2 and iNOS / NFκB / Pterostilbene / Resveratrol

Received: November 16, 2007; revised: april 30, 2008; accepted: May 1, 2008

1 Introduction

Resveratrol (*trans*-3,5,4′-trihydroxystilbene) occurring in fruits, such as grapes, peanuts, some *Vaccinium* berries, as well as in beverages derived from grapes including red wine, is the most extensively studied stilbene derivative. This compound was shown to inhibit the growth of tumor cells in several *in vitro* and *in vivo* systems and was established as a chemopreventive agent in rodent models (reviewed in ref. [1]).

Although resveratrol has been shown to target various intracellular signaling molecules in cultured cell lines [2], the molecular mechanisms underlying chemopreventive activity of resveratrol *in vivo* remain largely unresolved.

Transcription factors that participate in many cancer-related signal transduction pathways appear to be a very attractive target for chemoprevention. Although the relationship between inflammation and cancer has long been speculated, the molecular links between these two processes were discovered recently [3]. Due to these findings, the transcription factor nuclear factor-κB (NF-κB) has been implicated in the promotion of carcinogenesis [4]. One of the major target proteins controlled by NF-κB-driven trans-

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Abbreviations: AMC, 7-amino-4-methyl-coumarin; **AP-1,** activator protein-1; **COX-2,** cyclooxygenase-2; **IKK,** IκB kinase; **iNOS,** inducible nitric oxide synthase; **NF-κB,** nuclear factor-κB; **TMPD,** *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine; **TPA,** 12-*O*-tetradecanoylphorbol-13-acetate; **TRE,** TPA-responsive element (AP-1 binding consensus sequence)

activation are cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS). In the regulation of the expression of these enzymes, activator protein-1 (AP-1) transcription factor is also involved [5].

A recent study of Kundu and Surh [6] suggested that resveratrol targets IκB kinase (IKK) in blocking tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced NF-κB activation and COX-2 expression in mouse skin *in vivo*. Mouse skin is one of the best-defined animal models that properly reflect the concept of multistage carcinogenesis and allows the investigation of the mechanisms underlying carcinogenic as well as anticarcinogenic activities of chemicals.

The discovery of resveratrol as a cancer-preventive agent has prompted interest to search for other naturally occurring stilbenes with cancer preventing activity possibly more active and deprived of resveratrol's limitations such as poor bioavailability [7]. Grapes and berries of some *Vaccinium* species are some of the sources of pterostilbene (3,5-dimethoxy-4′-hydroxystilbene). This compound was found to be as effective as resveratrol in preventing carcinogen-induced preneoplastic lesions in mouse mammary organ culture model [8]. Intravenous administration of pterostilbene to mice inhibited metastatic growth of B16M-F10 melanoma cells in liver, a common site for metastasis development [9] and suppressed aberrant crypt foci formation in the azoxymethane-induced colon carcinogenesis model in rats [10]. Moreover, our recent studies demonstrated that pterostilbene is a more potent inhibitor of human recombinant CYP 1A1 catalytic activity than resveratrol [11]. This cytochrome P450 is involved in the activation of benzo[a]pyrene (B[a]P) leading to the formation of its ultimate carcinogenic metabolite, B[a]P-diol-epoxide.

This reactive B[a]P metabolite, besides forming DNA adducts critical for the initiation of the carcinogenesis, also induces transactivation of NF-κB [12]. Thus, the inhibition of CYP1A1 may be related to processes involved both in the initiation and the promotion stage of carcinogenesis.

The aim of our present study was to compare the effect of resveratrol and pterostilbene on TPA-induced activation of NF-κB and AP-1, and the expression and activity of COX-2 and iNOS in mouse epidermis. The results indicated that although both stilbenes affect the signaling pathways that control the expression of these enzymes, pterostilbene might be a more potent modulator of TPA-induced signal transmission.

2 Materials and methods

2.1 Materials

Resveratrol (purity 99%) and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma Chemicals (St. Louis, MO, USA). Pterostilbene was synthesized by partial methylation of *trans*-resveratrol as described previously [11]. Its structure was confirmed by UV, MS, and NMR spectroscopy.

Rabbit polyclonal c-Jun, c-Fos, IκBα, p65, IKKβ, COX-2 and iNOS antibodies, and anti-rabbit IgG-alkaline phosphatase (AP) were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-IκBα antibody was obtained from Cell Signaling Technology (Beverly, MA). Western blotting detection system was purchased from Bio-Rad Laboratories (Hercules, CA, USA). Colored protein M_r marker (Full Range Rainbow™) was purchased from Amersham Biosciences (Piscataway, NJ, USA).

All other compounds were readily available commercial products or their sources are indicated in procedures description.

2.2 Animals

Female Balb/c mice, 7 – 9 wk old (20 – 25 g), provided by the Nofer Institute of Occupational Medicine (Lódz, Poland), were housed in polycarbonate cages, containing hard-wood chip bedding. A standard pellet diet and distilled water were available without restriction. The mice were shaved on the dorsal side using surgical clippers 2 days before the treatment. TPA was applied to shaved skin (∼2 cm^2 patch) in the single dose of 10 nmol in 0.2 mL of acetone. Fifteen minutes before TPA application, the mice were treated with resveratrol or pterostilbene in the dose of 16 μmol in 0.2 mL of acetone. The dosage of stilbenes was selected based on our previous studies [13, 14]. A control group of mice was treated with acetone alone. For studying the time course of transcription factors activation, mice were sacrificed 1 – 12 h after TPA treatment. Otherwise the mice were killed at selected time points.

All experiments were conducted according to the Regional Ethics Committee's guidelines for animal experimentation.

2.3 NF-κB and AP-1 activation

NF-κB and AP-1 activation were assessed by an enzymatic immunoassay according to Renard *et al*. [15] using a commercial kit (TransAM assay; Active Motif, Carlsbad CA) and following the manufacturer's instructions. Activated NF-κB was measured in terms of the amount of NF-κB subunit p65, p50, and cRel contained in DNA binding complex extracted from the nuclear fractions isolated from mouse epidermis. The NF-κB consensus site (5′-GGGACTTTCC-3′) was immobilized on ELISA plates as bait.

Activated AP-1 was measured in terms of the amount of c-Jun and c-Fos subunits contained in DNA binding complex. The AP-1 consensus site (5′-TGAGTCA-3′, TPA-responsive element (TRE)) was immobilized on ELISA plates as bait.

Nuclear fractions were isolated from epidermis homogenates with the use of Nuclear/Cytosol Fractionation Kit (BioVision, Mountain View, CA, USA).

2.4 Isolation of subcellular fractions for Western blot

For the isolation of total protein, the scraped epidermis was placed in Total Protein Isolation Buffer (TPIB; 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM EDTA, 1 mM PMSF, 1 mM Na_3VO_4, 1 mM NaF), homogenized in ice-chilled glass homogenizer with Teflon pestle, lysed for 40 min on ice, and centrifuged for 30 min at $15\,000 \times g$. Supernatants were collected and stored at -70°C. For the separation of cytosolic and microsomal fractions, the scraped epidermis was placed in Cytosol and Microsomes Isolation Buffer (CMIB; 150 mM NaCl, 50 mM Tris-HCl, 2 mM EGTA, 1 mM DTT, 1 mM Na_3VO_4) containing protease inhibitors and homogenized in ice-chilled glass homogenizer with Teflon pestle. The cytosolic and microsomal fractions were prepared by differential centrifugation and stored at -70°C.

2.5 Western blot analysis

For the analysis of protein level, subcellular fractions or total protein fractions were boiled in loading buffer (2.7 M Tris-HCl, 20% SDS, 80% glycerol, 250 mM DTT, 0.01% bromophenol blue). Sixty micrograms of the sample protein were resolved on 10% polyacrylamide gels (Ready Gel 10% Resolving Gel Tris-HCl, BioRad Laboratories). For iNOS protein detection, 5% polyacrylamide gels were used (Ready Gel 5% Resolving Gel Tris-HCl, BioRad Laboratories). The resolved proteins were transferred to a PVDF membrane. The blot containing the transferred protein was blocked in blocking buffer (10% fat-free milk in PBS) followed by incubation with appropriate polyclonal or monoclonal primary antibody in PBS, and subsequently with anti-rabbit secondary antibodies conjugated with alkaline phosphatase 1:5000. After washing three times with PBS and two times with TBS (20 mM Tris-HCl, 500 mM NaCl; pH 7.4), blots were placed in 0.1 M Tris buffer (pH 9.5) and proteins were detected by Alkaline Phosphatase Conjugate Substrate Kit (BioRad Laboratories), scanned, and measured densitometrically with the use of BioRad Quantity One® software.

2.6 Immunoprecipitation and *in vitro* IKKβ activity assay

Cellular proteins (500 μg) were subjected to immunoprecipitation with anti-IKKα/β rabbit polyclonal antibody, and the immunocomplex was pulled down by shaking with protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology). The immunoprecipitate thus obtained was suspended in 40 μL of kinase reaction mixture, containing 50 mM HEPES (pH 7.5), 20 mM $MgCl_2$, 0.1 mM Na_3VO_4, 200 μM ATP, 10 mM β-glycerolphosphate, 2 mM DTT, and 2 μg of a peptide substrate for IKKβ (Cell Signaling Technology), and incubated for 30 min at 30°C. The reaction was stopped by addition of 50 μL of Concentrated Loading Buffer (312.5 mM Tris-HCl, pH 6.8, 250 mM DTT, 20% SDS, 50% glycerol, and 0.05% bromophenol blue) and boiled for 5 min. Thirty microliters of supernantant were separated on 10% SDS-PAGE gel (Ready Gel 10% Resolving Gel Tris-HCl, BioRad Laboratories), electroblotted to nitrocellulose membrane and after blocking with TBS-T (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.05% Tween-20) with 5% nonfat dry milk probed with either anti-phospho-IκBα which is specific for IκBα phosphorylated at Ser-32 or anti-IκBα which recognizes all IκBα. After rinsing with TBS-T, membranes were incubated with HRP-conjugated secondary antibody (Cell Signaling Technology) and visualized by LUMI-GLO reagent (Cell Signaling Technology) according to the manufacturer's instructions. Blots on X-ray films were evaluated densitometrically using BioRad Quantity One® software.

The enzyme activity was expressed as pmoles of P-IκBα produced *per* miligram of protein *per* minute.

2.7 Proteasome 20S activity assay

Excised skin was washed in ice-cold PBS buffer and epidermis was scraped-off and placed in ice-chilled tubes containing lysis buffer (50 mM HEPES, 5 mM EDTA, 5 mM DTT, 150 mM NaCl, 0.5 mM PMSF, 0.2 mM ATP, 1% Triton X-100, and 0.5% NP-40). After homogenization, the samples were lysed for 45 min on ice and centrifuged at $14\,500 \times g$ for 30 min. Supernatants were collected and stored at -70°C. Fourteen microliters of lysates were incubated for 60 min at 37°C with 20 μM of fluorigenic peptide substrate specific for 20S proteasome chymotrypsin-like activity Suc-Leu-Leu-Val-Tyr-AMC from Biomol (Plymouth Meeting, PA, USA) in a reaction mixture, containing 10 mM Tris-HCl (pH 7.4), 5 mM EDTA, 5 mM DTT, 0.03% SDS, and 0.2 mM ATP. The peptidase reaction was terminated by adding methanol. The fluorescence of the product, 7-amino-4-methyl-coumarin (AMC) was determined on a Hitachi F 2500 fluorescence spectrophotometer (λ ex 340 and λ em 440) and quantified based on comparison with the commercial AMC as a standard.

The chymotrypsin-like activity of 20S proteasome was expressed as pmoles of AMC hydrolyzed *per* miligram of protein *per* minute.

2.8 COX-2 activity assay

Excised skin was washed in Tris-buffer, pH 7.4 with heparin (0.1 M Tris-HCl, 1 mM EDTA, 16% heparin). Epidermis was scraped-off, suspended in 1 mL of Tris-buffer,

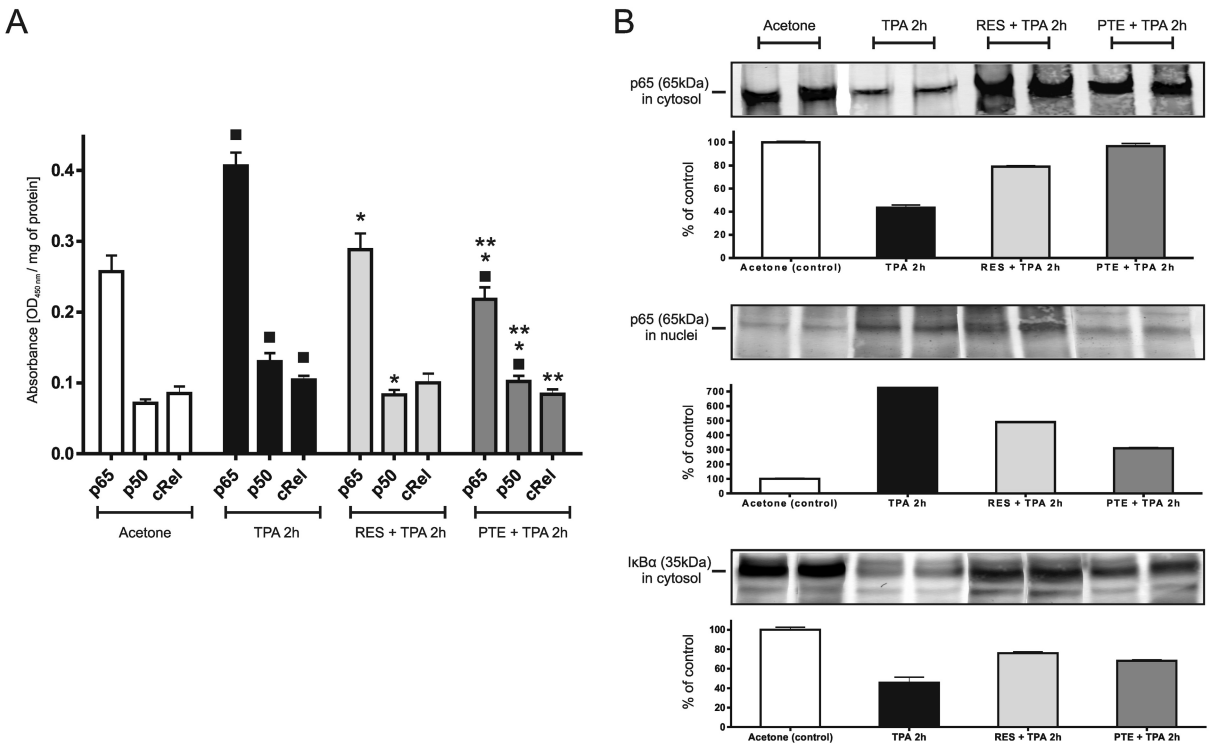

Figure 1. The effect of resveratrol and pterostilbene on the TPA-induced NF-κB activation in mouse epidermis. (A) Activated NF-κB was assessed in terms of the amount of NF-κB subunits p65, p50, or c-Rel contained in DNA binding complexes extracted from the nuclei isolated from epidermis and expressed as absorbance ($OD_{450\ nm}$ *per* mg of protein). Bars represent means ± SEM from three independent experiments. *Significantly different from TPA-treated group ($p < 0.01$). **Significantly different from resveratrol-treated group ($p < 0.05$). ■ Significantly different from acetone-treated group ($p < 0.01$). (B) Translocation of p65 from cytosol to nucleus and IκBα retention in cytosol were assayed by Western blot analysis. Data are representative of two independent experiments. Maximum variation between the two experiments was 10%.

pH 7.8 (0.1 M Tris-HCl, 1 mM EDTA), and homogenized in ice-chilled glass homogenizer with Teflone pestle. The homogenate was centrifuged 15 min at 14 500 × *g*. Collected supernatants were quickly frozen and stored at –70°C. The peroxidase activity of COX-2 was measured with the use of COX Activity Assay Kit from Cayman Chemical (Ann Arbor, MI, USA), according to manufacturer's protocol. Absorbance of oxidized *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) was measured at 590 nm. Application of COX-1 specific inhibitor allows to distinguish COX-2 activity.

The COX-2 activity was expressed as nmoles of oxidized TMPD produced *per* milligram of protein *per* minute.

2.9 Nitrite assay

The amount, of nitrite, an indicator of NO synthesis, was measured by Griess reaction [16].

Briefly, Griess reagent (1% sufanilamide, 0.1% naphtylethylenediamine, 2.5% phosphoric acid) was added to the same volume of epidermal cytosolic fraction (0.4 mL). After incubation at room temperature for 10 min, absorbance at 550 nm was measured and the concentration was calculated using sodium nitrite as a standard.

2.10 Statistical evaluation

The statistical analysis was performed by one-way ANOVA. The statistical significance between the experimental groups and their respective controls was assessed by Tukey's *post-hoc* test, with $p < 0.05$ considered significant.

3 Results

3.1 Effect of resveratrol and pterostilbene on NF-κB activation in mouse epidermis

The effect of topical application of resveratrol or pterostilbene on NF-κB activation is shown in Fig. 1. Application of resveratrol or pterostilbene (16 µmol *per* mouse), 15 min prior to TPA treatment resulted in a significant ($p < 0.01$) decrease in the amount of NF-κB subunits p65, p50, cRel contained in DNA binding complex of the nuclear fraction extracted from mouse epidermis 2 h after treatment (Fig.

Table 1. Inhibitory effects of resveratrol and pterostilbene on TPA-induced IKKβ activity in mouse epidermis

Treatment	Activity[a] pmol/min/mg protein
Acetone (control)	15.16 ± 0.60
TPA (10 nmol)	39.29 ± 2.36[b]
Resveratrol + TPA	9.48 ± 1.46[c]
Pterostilbene + TPA	12.10 ± 1.38[c]

a) IKKβ activity was assayed as described in Section 2 and is expressed as pmol of P-IκBα/min/mg protein. Control animals were treated with acetone instead of stilbenes or TPA. Values are mean ± SEM of three separate experiments. Statistical analysis was performed by ANOVA analysis followed by *post-hoc* Tukey's test.
b) Significantly different from control group at $p < 0.01$.
c) Significantly different from TPA-treated group at $p < 0.01$.

1A). Pterostilbene was more effective in inhibiting binding of NF-κB subunits, particularly p65. This stilbene also reduced p65 translocation from the cytosol to the nucleus (by two-fold) to a greater extent than resveratrol, in comparison with TPA-treated group of mice, and increased the retention of IκBα in the cytosol (Fig. 1B).

3.2 Suppression of IKKβ and proteasome 20S chymotrypsin-like activity in TPA-stimulated mouse skin

To get further insight into molecular mechanisms underlying inhibition of TPA-induced NF-κB activation by resveratrol and pterostilbene, we examined the effect of topically applied TPA alone or in combination with the stilbenes on the activity of IKKβ kinase and 20S proteasome – the active subunit of 26S proteasome, both involved in NF-κB activation. IKKβ phosphorylates IκBα, which is a signal for polyubiquitination and 26S proteasome-dependent degradation of IκBα subunit, allowing translocation of NF-κB to the nucleus. Western blot analysis of phosphorylated IκBα substrate peptide combined with LUMI-GLO reagent detection revealed the significant (in comparison to TPA-treated group) decrease of activity of IKKβ as a result of resveratrol or pterostilbene pretreatment (Table 1). Both stilbene derivatives reduced the 20S proteasome activity (Fig. 2), although resveratrol was slightly more effective.

3.3 Inhibitory effect of resveratrol and pterostilbene on AP-1 activation in mouse epidermis

Pretreatment of mouse skin with resveratrol or pterostilbene resulted in a decrease of c-Jun binding, while c-Fos was only slightly affected 4 h after TPA application. Again the effect of pterostilbene was more significant (Fig. 3A). c-Jun and c-Fos protein levels, however, were reduced to similar extent (Fig. 3B).

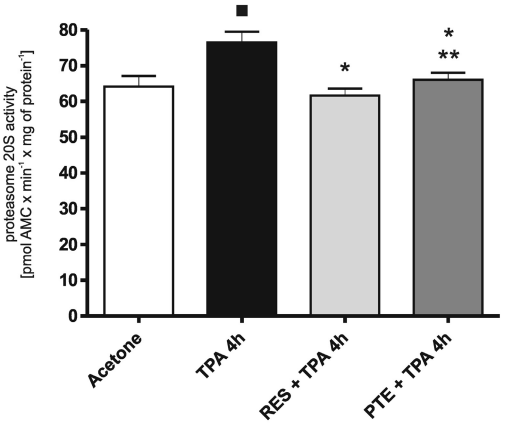

Figure 2. The effect of resveratrol and pterostilbene on the epidermal 20S proteasome. Proteasome activity is expressed as pmol of AMC released *per* miligram of protein *per* 1 min. Bars represent means ± SEM from three independent experiments. *Significantly different from TPA-treated group ($p < 0.01$). **Significantly different from resveratrol-treated group ($p < 0.05$). ■ Significantly different from acetone-treated control group ($p < 0.01$).

3.4 Inhibitory effect of resveratrol on TPA-induced COX-2 expression and activity in mouse epidermis

Topical application of TPA onto shaven backs of female Balb/c mice induced the expression of COX-2 protein maximally at 12 h (data not shown). Pretreatment with stilbenes 15 min prior to TPA application inhibited TPA-induced expression of COX-2 protein by 300% in comparison with TPA-treated group of mice (Fig. 4A) and reduced the enzyme activity by 56%, 12 h after treatment (Fig. 4B). No significant differences between the effect of resveratrol and pterostilbene on this enzyme activity were found.

3.5 Effect of resveratrol and pterostilbene on the expression and activity of iNOS

Our studies confirmed the earlier observation of Seo *et al*. [17] that topical application of 10 nmol of TPA also onto Balb/c female mice increased transiently the iNOS protein level with maximal expression observed at 2 h. Enzyme activity evaluated by the increase of nitrite production was also mostly enhanced at this time point. Pterostilbene reduced the iNOS protein level and enzyme activity by 180 and 45%, respectively, in comparison with TPA-treated group of animals. Resveratrol was less effective in inhibiting the enzyme expression and activity (Figs. 5A and B).

4 Discussion

The cancer chemopreventive property of resveratrol was first described by Jang *et al*. in 1997 [18], when it was dem-

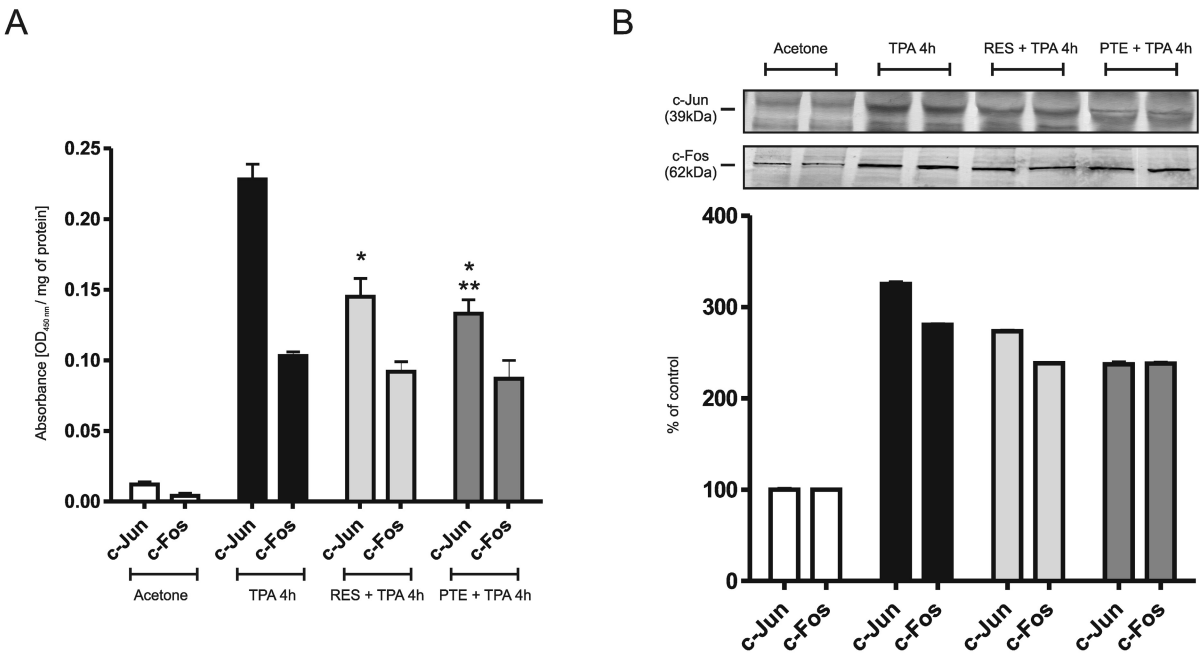

Figure 3. The effect of resveratrol and pterostilbene on the AP-1 activation in mouse epidermis. (A) Activated AP-1 was measured in terms of the amount of c-Jun or c-Fos subunits contained in DNA binding complexes extracted from the nuclei isolated from epidermis. Bars represent the means ± SEM from three independent experiments. *Significantly different from TPA-treated group ($p < 0.01$). **Significantly different from resveratrol-treated group ($p < 0.05$). (B) Protein extracts from mouse epidermis were assayed for c-Jun or c-Fos by Western blot analysis. The Western blot and bars is representative of two separate experiments with a maximal variation of 10%.

onstrated in the mouse skin model that this compound can inhibit both the initiation and the promotion stage of carcinogenesis and act as an antioxidant and antimutagen. Since then extensive studies were performed mostly *in vitro*, but also *in vivo* in other rodent models [19]. Our earlier studies showed that resveratrol in the dose of 16 μmol *per* mouse inhibited the formation the benzo[a]pyrene and 7,12-dimethylbenz[a]anthracene-DNA adducts in mouse skin. Several lines of evidence indicate that these polycyclic aromatic hydrocarbons may interfere with cellular signaling system by inducing or activating transcription factor NF-κB [12, 20]. Thus, the activation of this transcription factor by polycyclic aromatic hydrocarbons might be responsible for tumor induction in complete carcinogenesis protocol. In two-stage carcinogenesis mouse skin model, activation of NF-κB and AP-1 is stimulated by the application of phorbol ester, TPA. Modulation of TPA response might be considered as a part of anti-promotional activity in this experimental model.

Recent studies of Kundu *et al*. [1] have shown that topical application of resveratrol in the dose of 0.25 and 1 μmol significantly inhibited TPA-induced COX-2 expression by diminishing the activation of NF-κB *via* blockade of upstream kinase IKK signaling. Our present study confirmed these observations although the dose of resveratrol we have applied was higher and the techniques we used were different from that described by Kundu *et al*. [1]. In this regard, our studies showed that resveratrol in the dose of 16 μmol reduced the TPA-stimulated expression and activity of COX-2 in concert with a decrease in NF-κB active subunits translocation to the nucleus and binding to NF-κB consensus site. There was no clear correlation between COX-2 protein level and activity. This discrepancy, however, may result from the way of COX-2 activity normalization. Moreover, our study demonstrated that resveratrol also reduces, although to a lesser extent, the activity of 20S proteasome, the active subunit of 26S proteasome that is involved in the activation of NF-κB, and in many signaling pathways activated in cancer [21]. We also observed that the expression and activity of TPA-induced iNOS was diminished by resveratrol.

This observation is consistent with another finding of the same group indicating that NO can upregulate COX-2 in TPA-treated mouse skin through the activation of NF-κB. Because AP-1 is likely to play a role in regulating the induction of COX-2 and iNOS in mouse skin, we also determined the effect of resveratrol on the activation of this transcription factor. AP-1, in contrast to NF-κB, is minimally activated under normal physiological conditions, but is dramatically activated by various stimuli including phorbol esters [22]. Homodimers c-Jun/c-Jun and c-Jun/c-Fos heterodimers of AP-1 preferentially bind to the AP-1 consensus sequence TRE (TGAC/GTCA). The activity of AP-1 is regulated at the level of transcription of *c-jun* and *c-fos*

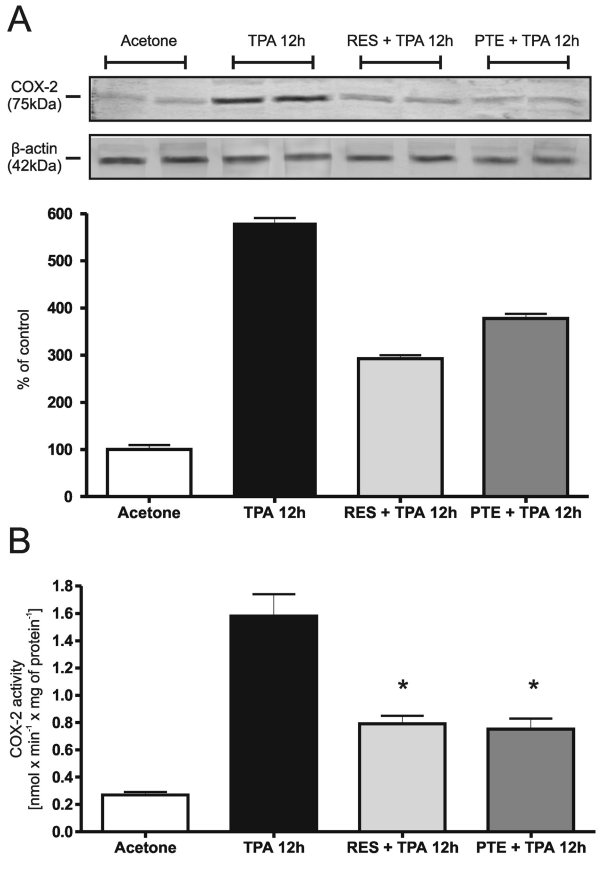

Figure 4. The effect of resveratrol and pterostilbene on mouse epidermal COX-2 protein expression and activity. (A) The blot and bars represent an average of two separate experiments with a maximal variation of 10%. (B) The peroxidase activity of COX-2 is expressed as nmol of oxidized TMPD produced *per* miligram of protein *per* minute. The values shown are means ± SEM from three independent experiments. *Significantly different from TPA-treated group ($p < 0.01$).

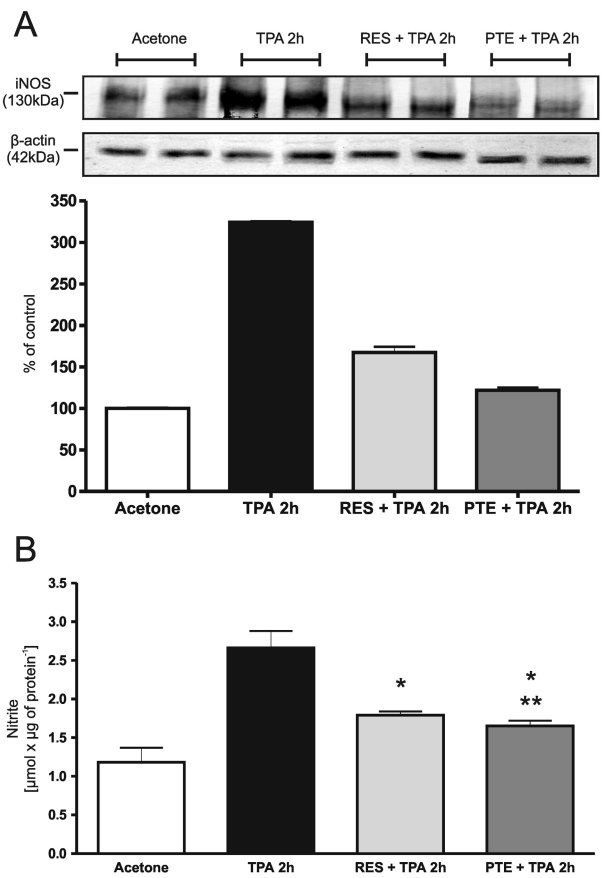

Figure 5. Effect of resveratrol and pterostilbene on the activation of iNOS in mouse epidermis. (A) iNOS protein expression was assessed by Western blot analysis. The blot and bars is representative of two independent experiments. (B) The amount of nitrite is expressed as micromole of sodium nitrite *per* mg of protein. The values shown are means ± SEM from three independent experiments. *Significantly different from TPA-treated group ($p < 0.01$). **Significantly different from resveratrol-treated group ($p < 0.05$).

genes by protein–protein interactions and also through post-translational modifications of Jun and Fos proteins [23, 24]. Our studies showed that resveratrol significantly reduced the binding of c-Jun to TRE site, while the binding of c-Fos was not affected. This observation is important since it was demonstrated that c-Fos is not the key protein responsible for mediating the activation of AP-1 in TPA-treated mouse skin. The expression of 5′-flanking region of *c-jun* expression is positively autoregulated through the binding of AP-1 to TRE sites on *c-jun* [25], and several lines of evidence indicate that AP-1 signaling mediated by c-Jun is important in mouse skin tumor promotion, while *c-fos* expression is significant in benign-to-malignant progression of mouse skin carcinogenesis.

Although resveratrol is considered as a very promising chemopreventive agent, its anticarcinogenic activity may be limited by its low bioavailability [26]. There are several reports indicating that chemical modifications of the stilbene backbone of resveratrol enhance the biological activities of its analogs. Thus, the most interesting finding of our current study is demonstrating that the naturally occurring resveratrol analog, pterostilbene is at least as potent an inhibitor of tumor promotion biomarkers as resveratrol. Moreover, pterostilbene more efficiently decreased the activation of NF-κB, AP-1, and iNOS expression.

This compound, which is one of the active constituents of blueberries, is a dimethylether analog of resveratrol. Substitution of hydroxy with methoxy group increases lipophilicity, which may have contributed to the improved bioavailability of this compound. This effect was demonstrated in the comparative studies of resveratrol and pterostilbene plasma levels [27]. The authors calculated a half-life of resveratrol and pterostilbene in mouse plasma after intravenous administration as ~10.2 and 77.9 min, respectively. Pterostilbene was found to be as effective as resveratrol in

preventing carcinogen-induced preneoplastic lesions in a mouse mammary organ culture model [8]. Additionally, intravenous administration of pterostilbene to mice inhibited metastatic growth of B16M-F10 melanoma cells in liver, a common site for metastasis development [9]. Recently, it was shown that administration of pterostilbene for 8 wk significantly suppressed the azoxymethane-induced formation of colorectal aberrant crypt foci (57%) in rats and multiple clusters of aberrant crypts (29%). Importantly, dietary pterostilbene also suppressed azoxymethane-induced colonic cell proliferation and iNOS expression. Inhibition of iNOS expression by pterostilbene was confirmed in cultured human colon cancer cells. Based on the results from our present study, inhibition of AP-1 and/or NF-κB might be responsible for this effect.

Our present study demonstrates the ability of pterostilbene administered directly to the skin, to inhibit the signaling cascade leading to COX-2 and iNOS induction by the tumor promoter. Since similar effect was found for the COX-2 inhibitor celecoxib [28], our study provides additional arguments for exploring the prospective clinical efficacy of topically applied COX-2 inhibitors for the management of human skin cancer as well as inflammatory disorders.

In summary, the results of our present study together with the earlier data suggest that pterostilbene, a compound present in blueberries, is of great interest for the prevention of cancer.

Supported by the Ministry of Science and Higher Education of Poland, grant 2P05F 049 26.

The authors have declared no conflict of interest.

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