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# Rosmarinic acid inhibits epidermal inflammatory responses: anticarcinogenic effect of *Perilla frutescens* extract in the murine two-stage skin model

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***Perilla frutescens*** **extract showed marked reduction on tumorigenesis in a murine, two-stage skin carcinogenesis model. In this model, cancer is initiated by application of 7,12-dimethylbenz[*a*]anthracene (DMBA) and promoted by application of 12-tetradecanoylphorbol 13-acetate (TPA). Following tumor initiation with DMBA, topical application of a perilla-derived fraction (PF) at doses of 2 mg/mouse/application resulted in significant inhibition of tumorigenesis. The efficacy of each fraction was correlated with rosmarinic acid (RA) and luteolin concentration. Topical application of perilla extract (PE) that contained 68% RA or an equivalent amount of commercially available RA showed nearly identical antiinflammatory activity 5 h after TPA treatment. Application of luteolin had less anti-inflammatory activity. Marked neutrophil infiltration was observed in TPA-challenged skin by histological examination using hematoxylin–eosin. This change was greatly reduced by pre-treatment with PE or RA. Myeloperoxidase activity, a marker of neutrophil recruitment, was also increased in TPA-challenged skin and was significantly decreased in the PE and RA treated groups. Intercellular adhesion molecule 1 and vascular cell adhesion molecule-1 mRNA expression levels were reduced by pre-treatment with PE or RA. TPA-induced increases in synthesis of the chemokines KC and macrophage inflammatory protein-2 were significantly decreased by pre-treatment with PE or RA. Prostaglandin E2 and leukotriene B4 levels were slightly increased 5 h after TPA treatment. These levels were only numerically decreased in the PE and RA treated groups. However, induction of cyclooxygenase-2 mRNA expression was obviously reduced by pre-treatment with PE or RA. Reactive oxygen radical production, detected as thiobarbituric acid reactive substance and lipid peroxide, by double treatment of TPA was reduced by pre-treatment with PE or RA. Production of 8-hydroxy-2′deoxyguanosine, which was detected immunohistochemically, was also induced by double treatment with TPA. This adduct was barely visible in PE or RA treated mice. Thus, we conclude that part of the anticarcinogenic effects of *P.frutescens* extract is due to RA via two independent mechanisms: inhibition of the inflammatory response and scavenging of reactive oxygen radicals.**

## Introduction

Results of epidemiological studies have shown that frequent consumption of plant-derived foods such as vegetables, fruits, tea and herbs, is associated with a lower risk of a variety of cancers (1–3). This suggests that cancer preventive substances exist in plant-derived foods. Many reports have suggested that plant-derived phytochemicals prevent cancer in experimental animal models (4–6). Specifically, antioxidants such as flavonoids, phenolic acid and proanthocyanidins have shown potent anticancer activity in two-stage skin papilloma models using 7,12-dimethylbenz[*a*]anthracene (DMBA) and 12-tetradecanoylphorbol 13-acetate (TPA) (7–9).

Rosmarinic acid (RA) is found in many medicinal species of the plant Lamiaceae (Figure 1) including basil, sage, rosemary, mint (10) and *Perilla frutescens*, one of the most popular garnishes in Japan. Extract of perilla appears to be a strong anti-inflammatory agent as it inhibits mast cell release of histamine (11), inhibits lipoxygenase activity (12) and is an antioxidant (13–15). Recently, we reported that perilla extract (PE) and RA reduce liver injury induced by D-galactosamine and LPS in mouse, due to scavenging of superoxide molecules produced by Kupffer cells and inhibition of peroxynitrite formation induced by inducible nitric oxide synthase (iNOS) (16).

Previous reports suggest that the body's response to cancer has many parallels with the inflammatory response (17,18). For example, inflammatory cytokine networks may influence survival, growth, mutation, proliferation, differentiation and movement of both tumor and stromal cells (18). In addition, cyclooxygenase-2 (COX-2), a key enzyme in the inflammatory response, is thought to affect carcinogenesis indirectly via its modulatory effect on the inflammatory response and the immune system (19,20).

In the present study, we examined the effects of PE in a two-stage, mouse skin papilloma model system. To further elucidate these effects, we also compared the effects of PE, RA and luteolin on edema formation induced TPA.

**Abbreviations:** COX-2, cyclooxygenase-2; DMBA, 7,12-dimethylbenz[*a*]anthracene; ICAM-1, intercellular adhesion molecule 1; LPO, lipid peroxide; $LTB_4$, leukotriene B4; MIP-2, macrophage inflammatory protein-2; MPO, myeloperoxidase; NFκB, nuclear factor κB; 8OH-dG, 8-hydroxy-2′deoxyguanosine; PE, perilla extract; PF, perilla fraction; $PGE_2$, prostaglandin E2; PKC, protein kinase C; RA, rosmarinic acid; TBARS, thiobarbituric acid reactive substance; TFA, trifluoroacetic acid; TPA, 12-tetradecanoylphorbol 13-acetate; VCAM-1, vascular cell adhesion molecule-1.

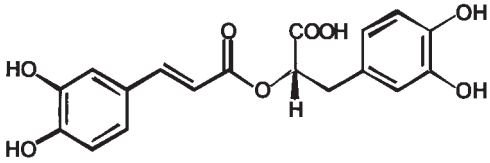


**Fig. 1.** Chemical structure of RA.

## Materials and methods

*Mice*

Seven- to nine-week-old male, BALB/c mice purchased from Crea Japan (Tokyo, Japan) were used for this study. Mice were kept in a controlled environment at 23°C and 55% humidity under a 12 h light/dark cycle. The Animal Committee of Meiji Seika Kaisha Health and Bioscience Laboratory approved this study and mice received care under guidelines provided by this committee.

*Chemicals*

RA was obtained from Extrasynthase (Genay, France). TPA and DMBA were obtained from Sigma (St Louis, MO). Anti-8-hydroxy-2′deoxyguanosine (8OH-dG) antibody was obtained from the Japanese Institute for the Control of Aging (Shizuoka, Japan). All other chemicals were of reagent grade.

The perilla fraction (PF) was prepared as follows. Fresh perilla leaves were extracted with 1% w/v citric acid at 90°C for 30 min. The extract was concentrated and applied to a Diaion HP2MG open column (Mitsubishi Kasei Co., Tokyo, Japan). The column was step-wise eluted by 0.1% w/v trifluoroacetic acid (TFA) in distilled water and methanol. Fractions eluted by 0.1% w/v TFA containing 20, 50 or 80% v/v methanol were collected, freeze-dried and used for experiment 1. The concentration of polyphenols, including caffeic acid, protocatecuic acid, RA and luteolin, was determined by HPLC using a Develosil HG-5 column (Nomura Chemical Co., Aichi, Japan) with solvents A (0.1% v/v TFA in distilled water) and B (0.1% TFA v/v in acetonitrile), under the following conditions: 10–50% linear gradient of A in B; flow rate 0.8 ml/min; detection 280 nm. The concentration of polyphenols in each fraction is shown in Table I.

The PE used in experiments 2 and 3 was prepared as follows. The extract derived from fresh perilla, as described above, was concentrated and then mixed with *n*-butanol. The resulting *n*-butanol layer was dried and the residue was dissolved in distilled water. This solution was applied onto a Diaion HP2MG column and washed with distilled water. The fraction eluted by 0.1% w/v TFA containing 60% v/v methanol was collected and freeze-dried. The RA concentration in this fraction was 68% w/w. Concentration of luteolin, luteolin-7-*O*-glucoside, protocatechuic acid and caffeic acid were all under the detectable limit (>50 ng/ml).

*Experimental procedure*

All test chemicals and TPA were dissolved in ethanol, and 20 μl of this solvent was topically applied to each animal.

*Experiment 1*. Eighty mice were divided into four groups as shown in Figure 2a. All mice were treated topically on dorsal, saved skin with 50 μg/20 μl/mouse DMBA dissolved in acetone. One week later, animals were treated topically with PF 1, 2 or 3 at a dose of 2 mg/mouse or control vehicle 30 min before treatment with 2 nmol TPA/mouse. These treatments were repeated twice weekly until termination of the experiment at 20 weeks after DMBA treatment. Skin tumor formation was recorded weekly and tumors >1 mm in diameter were included in the cumulative total if they persisted for 2 or more weeks.

*Experiment 2*. Mice were divided into nine groups: vehicle, TPA-challenged control or TPA-challenged and treated with 2.0 mg/mouse PE or 0.25 or 0.5 mg/mouse luteolin, which is the maximum dissolved dosage, or 0.25, 0.5, 1.0 or 1.35 mg/mouse RA (Figure 2b). RA (1.35 mg/mouse) was administered in ethanol at an amount equivalent to 2.0 mg/mouse of PE. PE, RA and luteolin treatments were applied 30 min before treatment with 2 nmol TPA/mouse. Animals were killed under anesthesia 1 or 5 h after TPA application. A mouse ear punch was obtained with a 5 mm cork bowler and was subsequently weighed. Punches were kept at −80°C until analysis.

**Table I.** Composition of polyphenol fraction derived from *P.frutescens*

| | Concentration w/w% | | |
|---|---|---|---|
| | Fraction 1 | Fraction 2 | Fraction 3 |
| RA | ND | 10.70 | 40.05 |
| Protocatechuic acid | 0.10 | 0.10 | ND |
| Caffeic acid | ND | 0.60 | 0.16 |
| Luteolin | 0.30 | 0.40 | 1.13 |

ND, not detected.

*Experiment 3*. Mice were divided into nine groups: vehicle, TPA single challenged, TPA double challenged, TPA double challenged and double treated with PE (2.0 mg/mouse) or RA (1.35 mg/mouse), TPA double challenged and treated with PE or RA 30 min prior to the first or second TPA treatment (Figure 2c). TPA, PE and RA treatments were performed as described above. The second TPA treatment was performed 24 h after the first TPA treatment. Animals were killed under anesthesia 1 h after the second TPA application.

*Histological evaluation*

The harvested ears were fixed in 3% buffered formalin and embedded in paraffin. Five-micrometer sections were affixed to slides, deparaffinized and stained with hematoxylin and eosin to assess morphological changes. The production of 8OH-dG was detected by immunohistochemical localization using an anti-8OH-dG monoclonal antibody. Briefly, deparaffinized slides were placed in blocking reagent containing $H_2O_2$ for 45 s to quench endogenous peroxidase. The sections were then blocked with 10% normal goat serum for 1 h. After blocking, 1 μg/ml 8OH-dG was incubated with the sections for 1 h at room temperature in a moist chamber. Incubation was followed by the addition of a biotinylated secondary antibody and streptavidin–peroxidase conjugate. The slides were incubated with 3,3-diaminobenzidine tetrahydrochloride to detect 8OH-dG production, and were then counterstained with hematoxylin.

*Analyses*

Myeloperoxidase (MPO) activity was determined according to the method of Bradley *et al*. (21). Tissue thiobarbituric acid reactive substance (TBARS) levels were determined according to the method of Ohkawa *et al*. (22) using tetramethoxypropane (Wako Pure Chemical Industries, Tokyo, Japan). Lipid peroxide (LPO) content was measured spectrophotometrically using a commercially available kit (Determiner LPO, Kyowa Medics Co., Tokyo, Japan). KC, macrophage inflammatory protein-2 (MIP-2), prostaglandin E2 ($PGE_2$) and leukotriene B4 ($LTB_4$) concentrations were analyzed by ELISA using a kit purchased from R&D Systems (Minneapolis, MN). Semi-quantitative analysis of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and COX-2 was performed using RT–PCR. Total RNA was extracted from mice livers using TRIzol (Invitrogen, Carlsbad, CA). cDNA synthesis was performed using the Reverse Transcription System (Promega, Madison) according to the manufacturer's instructions. PCR primers (Sawady Technology, Tokyo, Japan) were as follows: ICAM-1 sense primer, 5′-TCG GAG GAT CAC AAA CGA AGC-3′ corresponding anti-sense primer, 5′-AAC ATA AGA GGC TGC CAT CAC G-3′; VCAM-1 sense primer, 5′-CCT CAC TTG CAG CAC TAC GGG CT-3′ and the corresponding anti-sense primer, 5′-TTT TCC AAT ATC CTC AAT GAC GGG-3′; COX-2 sense primer, 5′-ACT CAC TCA GTT TGT TGA GTC ATT C-3′ and the corresponding anti-sense primer, 5′-TTT GAT TAG TAC TGT AGG GTT AAT G-3′; β-actin sense primer, 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′ and the corresponding anti-sense primer, 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′. PCR conditions for ICAM-1and VCAM-1 were 35 cycles of denaturation at 94°C for 15 s, annealing at 55°C for 30 s and extension at 72°C for 45 s. PCR conditions for COX-2 were 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 60 s and extension at 72°C for 90 s. PCR conditions for β-actin were 30 cycles for denaturation at 95°C for 30 s, annealing at 60°C for 1 min and extension at 72°C for 2 min. A final extension reaction was then carried out at 72°C for 5 min. The predicted size of ICAM-1, VCAM-1, COX-2 and β-actin were 431, 441, 583 and 348 bp, respectively. PCR products were electrophoresed on 2.0% agarose gels. Ethidium bromide-stained gels were visualized under UV light (Bio-Rad, Hercules, CA) and each product was normalized to give an equivalent signal from the β-actin.

*Statistical analysis*

The data were expressed as the mean plus or minus the standard error. Analyses were performed using SPSS statistical software. When ANOVA revealed $P < 0.05$, the data were further analyzed by Sheffe's multiple range test. Differences were considered statistically significant at $P < 0.05$.

## Results

### *Antitumor-promoting activity of PF*

Topical application of PF prior to TPA application resulted in significant inhibition of TPA tumor promotion in DMBA-initiated BALB/c mouse skin (Figure 3). The preventive effect of these agents was evident as a reduction in tumor incidence (Figure 3a) and tumor multiplicity (Figure 3b). The extent of tumor reduction was correlated with RA and luteolin concentrations.

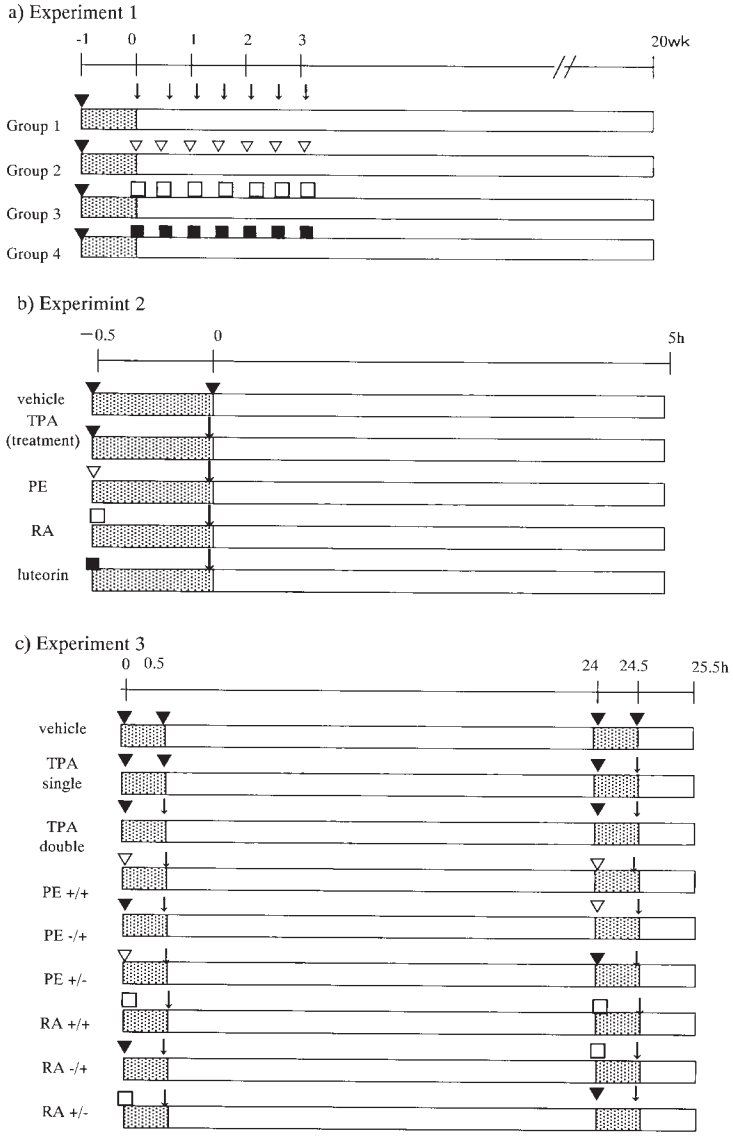


**Fig. 2.** Experimental procedure of murine two-stage skin papilloma model and TPA induced ear edema inflammation model. (**a**) Filled triangle, DMBA 50 μg/mouse; downwards arrow, TPA 2 nmol/mouse; empty triangle, TPA with PF-1, 2 mg/mouse; empty square, TPA with PF-2, 2 mg/mouse; filled square, TPA with PF-3, 2 mg/mouse topical application. (**b**) Filled triangle, ethanol 20 μl/mouse; downwards arrow, TPA 2 nmol/mouse; empty triangle, PE 2 mg/mouse; empty square, rosmarinic acid 0.25, 0.5, 1.0 or 1.35 mg/mouse; filled square, luteolin 0.25 or 0.5 mg/mouse topical application. (**c**) Filled triangle, ethanol 20 μl/mouse; downwards arrow, TPA 2 nmol/mouse; empty triangle, PE 2 mg/mouse; empty square, rosmarinic acid 1.35 mg/mouse topical application.

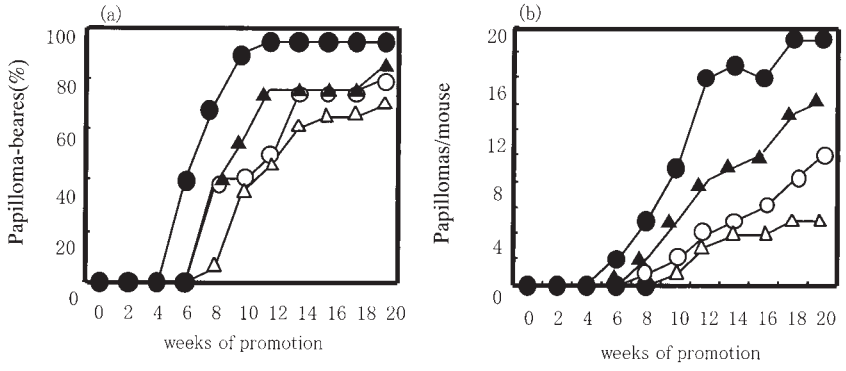


**Fig. 3.** Inhibitory effect of polyphenolic fractions derived from *P.frutescens* on TPA tumor promotion in DMBA-initiated mouse skin. One week after topical application of DMBA (50 μg/mouse), 2 nmolTPA/mouse was applied twice weekly with or without (filled circle) prior application of 2 mg/mouse of each fraction (filled triangle, fraction 1; empty circle, fraction 2; empty triangle, fraction 3). The percentage of mice with tumors (**a**) and the number of tumors per mouse (**b**) are shown.

*Anti-inflammatory effects of PE and RA*

As shown in Figure 4, ear weight was markedly increased by TPA treatment. This change was suppressed by pre-treatment with 2.0 mg PE/mouse. RA also reduced ear weight in a dose-dependent manner. However, luteolin did not reduce such ear weight gain of 0.25 and 0.5 mg/mouse. Treatment with 1.35 mg RA/mouse, a dose equivalent to 2.0 mg PE/mouse, decreased ear weight to a similar degree as PE treatment. Histological examination of ear sections from TPA-challenged animals revealed skin edema with massive neutrophil infiltration (Figure 5). In the PE (2.0 mg/mouse) and RA (1.35 mg/mouse) groups, slight edema was present with only a relatively small amount of neutrophil infiltration. Myeloperoxidase (MPO) activity in the ear was correlated with the histological changes shown in Figure 6. Specifically, TPA treatment increased MPO activity and this activity was significantly reduced in the PE and RA groups.

*Induction of adhesion molecule mRNA in mouse ears*

mRNA expression in mice ears was analyzed by RT–PCR 1 and 5 h after TPA treatment. PCR products with lengths of 431 bp corresponding to ICAM-1 were detected in TPA-treated mice as shown in Figure 7. ICAM mRNA expression was slightly reduced by PE (2.0 mg/mouse) and RA (1.35 mg/mouse) 1 and 5 h after treatment. As shown in Figure 7, VCAM-1 mRNA expression was markedly increased by TPA treatment. VCAM-1 expression was barely detectable in these conditions, suggesting that PE and RA treatment blocked this induction. The effect was strongest 1 h after TPA treatment.

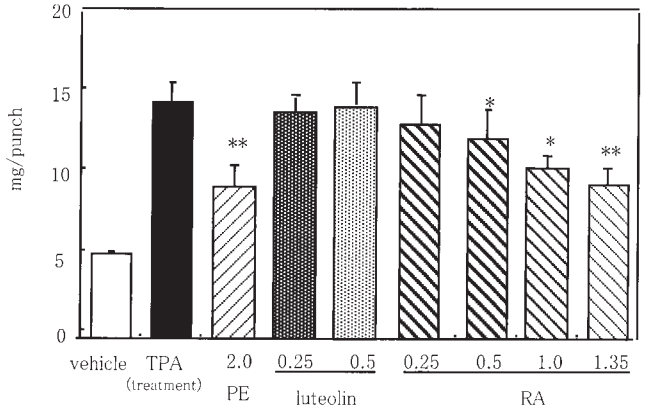


**Fig. 4.** Effects of prior application of PE, RA or luteolin on TPA-induced ear edema. Significant differences from TPA (treatment) are indicated; $^{*}P < 0.05$; $^{**}P < 0.01$ by ANOVA and Sheffe's multiple range test.

*Chemokine expression in mouse ears*

Chemokine concentrations are shown in Figure 8. KC was markedly increased by TPA challenge (Figure 8a). This elevation was significantly reduced in the PE (2.0 mg/mouse) and RA (1.35 mg/mouse) groups. Similar data were obtained for MIP-2 (Figure 8b).

*Eicosanoid concentration and induction of cyclooxygenase mRNA in mouse ears*

$PGE_2$ and $LTB_4$ levels are shown in Figure 9a and b. TPA challenge appeared to increase the concentration of both eicosanoids relative to the vehicle group and PE and RA treatment appeared to block this induction. However, these differences did not reach statistical significance. A PCR product that was 583 bp in length and corresponded to COX-2 was detectable in the control group 5 h after TPA challenge but was not detectable in mice pre-treated with PE or RA.

*TBARS and LPO concentration in mouse ears*

While a single TPA challenge had no effect on TBARS and LPO levels, a double TPA challenge resulted in a significant elevation of these markers of oxidative stress (Figure 10a and b). PE or RA treatment significantly inhibited this elevation. The expression levels of TBARS and LPO were also reduced by both PE and RA—even when mice received only one treatment with these agents (prior to the first or second application of TPA).

*8OH-dG expression in mouse ears*

Figure 11 shows immunohistochemical staining for 8OH-dG (light brown staining). No 8OH-dG was detectable in the unchallenged group. However, 8OH-dG adducts were observed in the cell nuclei from TPA double-challenged but not TPA single-challenged mice. No staining was present in PE or RA treated mice, indicating that PE and RA blocked 8OH-dG expression.

## Discussion

In this study, we evaluated the effects of *Perilla frutescens* extract in the mouse two-stage skin carcinogenesis model. This extract has been reported to have potent anti-inflammatory effects (11,12). However, the identity of the anti-inflammatory component of PE is unclear, because the purple leaf of the plant contains numerous components including anthocyanins (23,24), flavonoids and phenolic acids (25). We observed anticarcinogenic activity in all PF, although there was a difference in efficacy between the fractions that correlated with RA and luteolin concentrations. We also compared the effects of PF containing commercially available, reagent grade RA

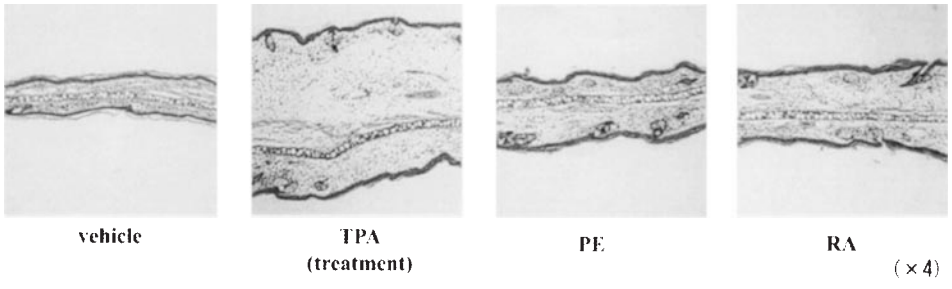


**Fig. 5.** Histological effects of prior application of 2.0 mg PE/mouse or 1.35 mg RA/mouse on TPA-induced ear edema.

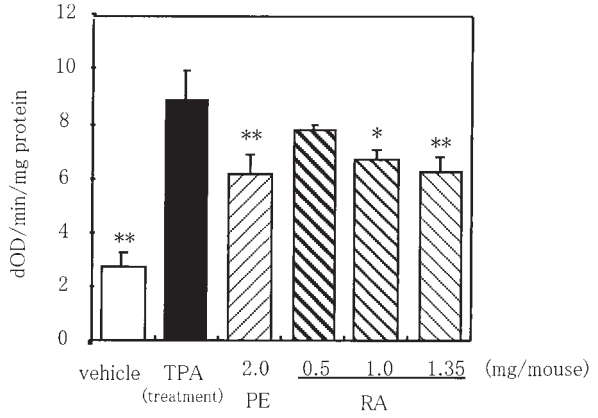


**Fig. 6.** Effects of prior application 2.0 mg PE/mouse and/or 1.35 mg RA/mouse on MPO activity. Significant differences from TPA (treatment) are indicated; $^{*}P < 0.05$; $^{**}P < 0.01$ by ANOVA and Sheffe's multiple range test.

and luteolin on TPA-induced mouse ear edema. RA is more effective than luteolin in inhibiting the TPA-induced inflammatory response, as shown in Figure 3.

We show that pre-treatment with PE and RA reduced TPA-induced neutrophil infiltration and elevated MPO activity. To investigate the mechanism that is responsible for inhibiting neutrophil infiltration, we analyzed mRNA expression of the adhesion molecule ICAM-1, which is primarily involved in interactions with $\beta_2$ integrins during inflammatory responses (26). It has been reported that ICAM-1 protein is localized to epidermal keratinocytes and vascular endothelium in TPA-treated skin (27). In our study, TPA induced ICAM-1 mRNA was reduced in PE and RA pre-treated mice. It has been reported that VCAM-1, a cytokine-inducible member of the immunogloblin superfamily that binds to the integrin VLA-4, is also induced by TPA treatment in endothelial cells (28,29). Here, reduction of VCAM-1 mRNA expression was observed following pre-treatment with PE or RA.

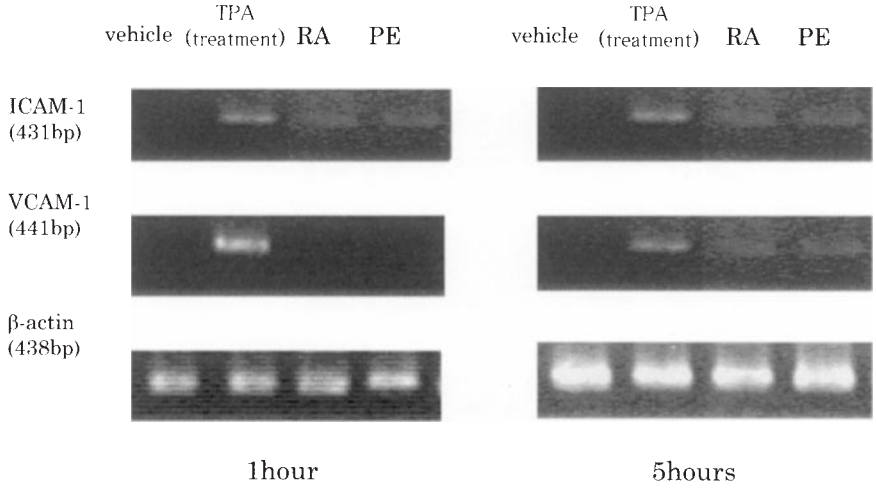


**Fig. 7.** Effect of prior application of 2.0 mg PE/mouse and/or 1.35 mg RA/mouse on VCAM-1 and ICAM-1 mRNA expression in mouse ears 1 and 5 h after topical application of TPA.

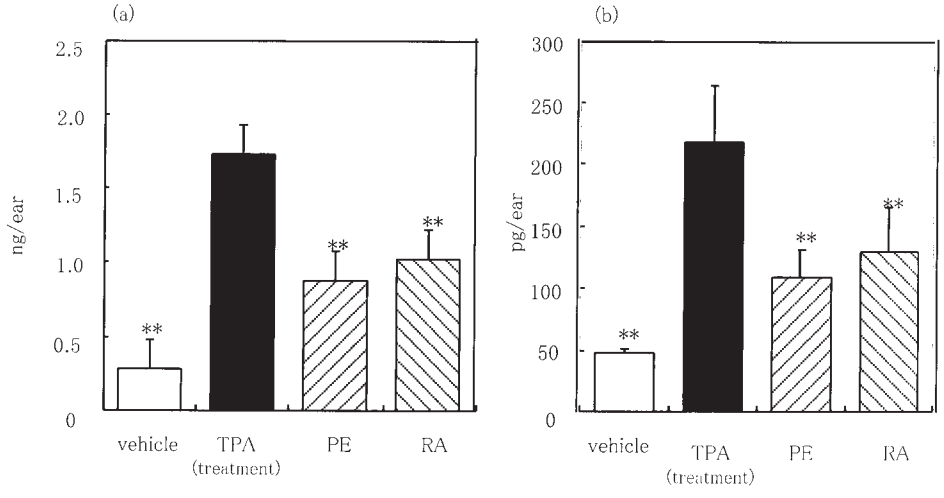


**Fig. 8.** Effect of prior application of 2.0 mg PE/mouse and/or 1.35 mg RA/mouse on TPA induced chemokine expression. KC (**a**) and MIP-2 (**b**) levels in mouse ears 5 h after topical application of TPA were determined by ELISA. Significant differences from TPA (treatment) are indicated; $^{*}P < 0.05$; $^{**}P < 0.01$ by ANOVA and Sheffe's multiple range test.

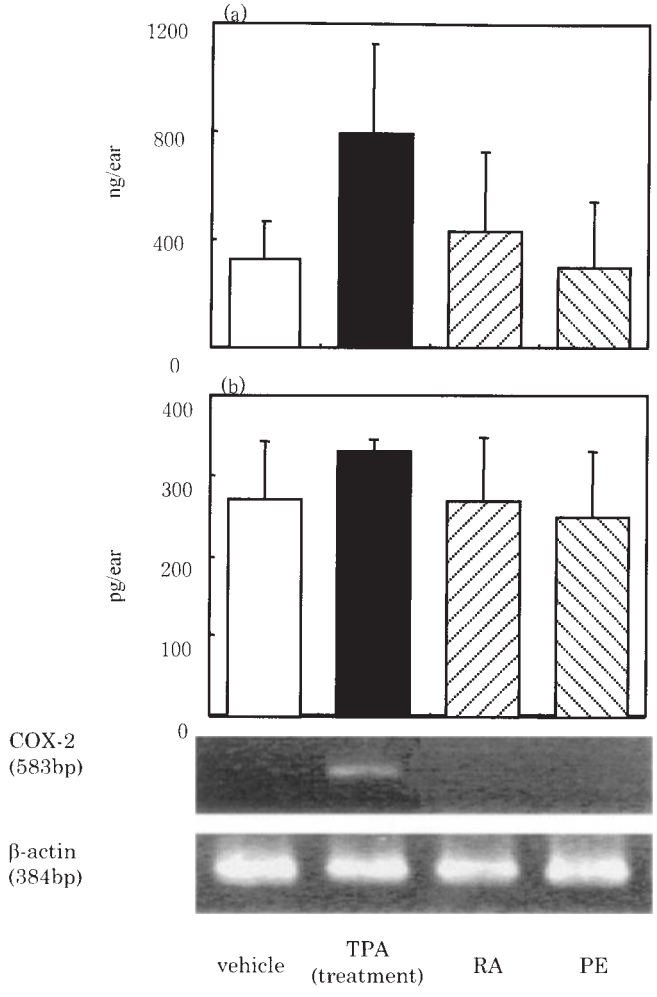


**Fig. 9.** Effect of prior application of 2.0 mg PE/mouse and/or 1.35 mg RA/mouse on TPA-induced $PGE_2$ (**a**) and $LTB_4$ (**b**) protein levels and COX-2 mRNA levels. Mouse ears were collected 5 h after topical application of TPA. $PGE_2$ and $LTB_4$ levels were determined by ELISA. COX-2 mRNA expression was detected by RT–PCR.

The TPA induced increased levels of the pro-inflammatory chemokine KC, a homolog of IL-8, was significantly reduced by pre-treatment with PE or RA. Many studies have shown that IL-8 is an important endothelial (30) and epithelial (31,32) derived inflammatory mediator that induces neutrophil chemotaxis and stimulates neutrophil transmigration. Protein kinase C (PKC) induces up-regulation of IL-8 mRNA and protein expression (33,34). We also found that expression of MIP-2, a homolog of the human CXC chemokine growth related oncogene (GRO) (35), was also increased by TPA treatment. Mouse MIP-2 has been characterized as a potent chemoattractant and is functionally equivalent to human IL-8 (36,37). Other investigators have shown that TPA-induced ear hyperplasia accompanied by MIP-2 up-regulation is clearly present in transgenic mice that overexpress PKC-α in the epidermis (38). A significant reduction in MIP-2 elevation was observed in both the PE and RA groups.

Epidemiological and experimental studies indicate that prostaglandins play a role in carcinogenesis (39,40). We found that treatment with PE and RA tended to decrease the TPA-induced increase in $PGE_2$ synthesis. Previous studies have shown that $PGE_2$ promotes cell proliferation (41) and prevents apoptotic cell death (42), activities that enhance cancer promotion. Furthermore, non-steroidal anti-inflammatory drugs, which decrease the production of prostaglandins, reduce the incidence and progression of tumors in both animal models and in cancer patients (43–45). In this study, COX-2 mRNA expression induced by TPA was eliminated when animals were pre-treated with PE or RA. Thus, reduction in prostaglandin synthesis may be a mechanism by which perilla and one of its effective components, RA, inhibit carcinogenesis. On the other hand, lipoxygenase-catalyzed products also have a profound influence on the development and progression of cancers (46). $LTB_4$, a biologically active metabolite of arachidonic acid, has been implicated in inflammatory disease and is one of the most potent chemoattractants and leukocyte activators (47). Moreover, RA, and its methylester, derived from perilla seed reportedly inhibit 5-lipoxygenase and 12-lipoxygenase activity (48,49) *in vitro*. In this study, $LTB_4$ appeared to be increased by TPA stimulation, and this small elevation was blocked by PE or RA treatment; however, these changes did not reach statistical significance.

Several previous studies have suggested an anti-inflammatory effect of PE. Yamazaki *et al.* (50) reported that PE administration reduced increases in plasma TNFα induced by i.v. injection of muramyldipeptide in mice. It has also been reported that induction of iNOS and NO production by cultured murine mesangial cells induced by interferon-γ and TNFα was reduced by the addition of PE (51). In our experiments, RA and PE reduced expression of cell adhesion molecules and COX-2 and inhibited the synthesis of CXC chemokines. The anti-inflammatory effect of RA in perilla is probably due to inhibition of these various inflammatory mediators.

Previous reports have shown that the transcription factor nuclear factor κB (NF-κB) is a central mediator of the immune response as it regulates the expression of pro-inflammatory enzymes, cytokine receptors and cell adhesion molecules. In this study we showed that RA inhibits the expression of important inflammatory mediators including ICAM-1, VCAM-1, KC, MIP-2 and COX-2. Many reports have indicated that regulation of gene expression of these mediators occurs via NF-κB (52–57). In contrast, PKC, which is induced by various agents that stimulate the immune response, is a strong activator of NF-κB (58). Several reports suggest that the properties of plant-derived phenolic compounds such as quercetin, myricetin and resvertorol may be mediated through downregulation of the NF-κB pathway (59,60). In addition, the novel antioxidant α-tocopherol inhibits PKC in various cell types (61). According to the results of this study, it seems that RA reduces TPA induced inflammatory responses by inhibiting the PKC/NF-κB pathway. Further study is needed to elucidate the molecular mechanism of the anti-inflammatory effects of RA.

It has been reported that double treatment with phorbol esters induces oxidative stress in mouse skin (62,63). PKC activation of recruited neutrophils in the inflamed area induced by the second application of TPA could have activated NADPH oxidase and acted as a major source of reactive oxygen in this model (64). Oxidized DNA levels in skin are also reportedly elevated by this treatment (65,66). In this study, pre-treatment with RA or PE in TPA application groups significantly reduced TBARS and LPO levels. In addition, immunohistochemical detection of 8OH-dG, one of the major markers of oxidative DNA damage, was inhibited in all RA

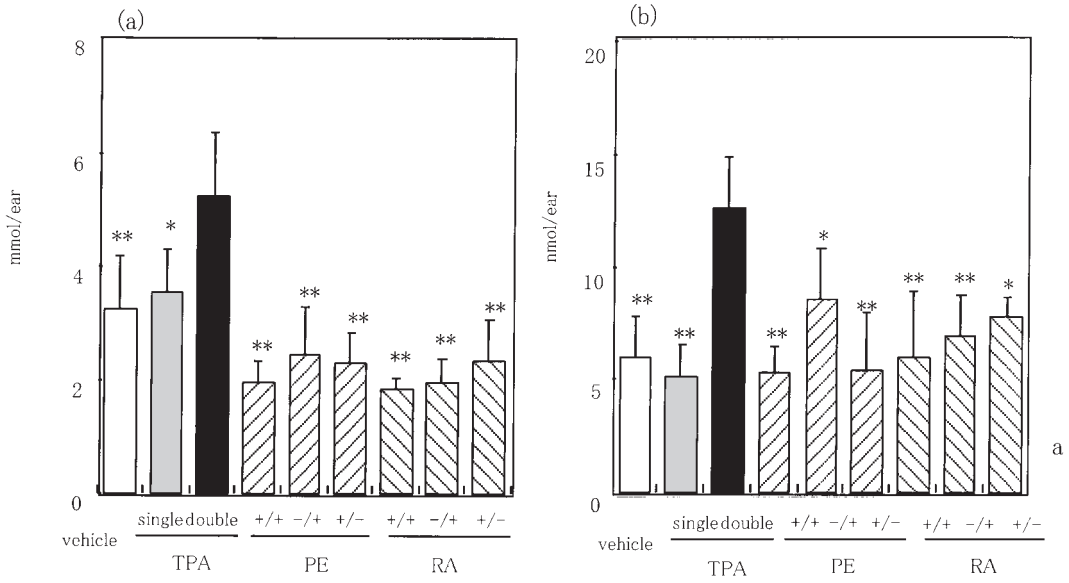


**Fig. 10.** Effect of prior application of PE or RA on double TPA treatment-induced production of TBARS (**a**) and LPO (**b**). Vehicle (TPA untreated and RA/PE untreated), TPA single challenged and untreated (TPA single), TPA double challenged and untreated (TPA double), TPA double challenged and double treated (+/+), TPA double challenged and RA/PE treated prior to first TPA treatment (+/−), TPA double challenged and RA/PE treated prior to the second TPA (−/+) treatment with 2.0 mg PE/mouse or 1.35 mg RA/mouse. The second TPA treatment was performed 24 h after the first TPA treatment. Animals were killed under anesthesia 1 h after the second TPA application. Significant differences from control are indicated; $^{*}P < 0.05$; $^{**}P < 0.01$ by ANOVA and Sheffe's multiple range test.

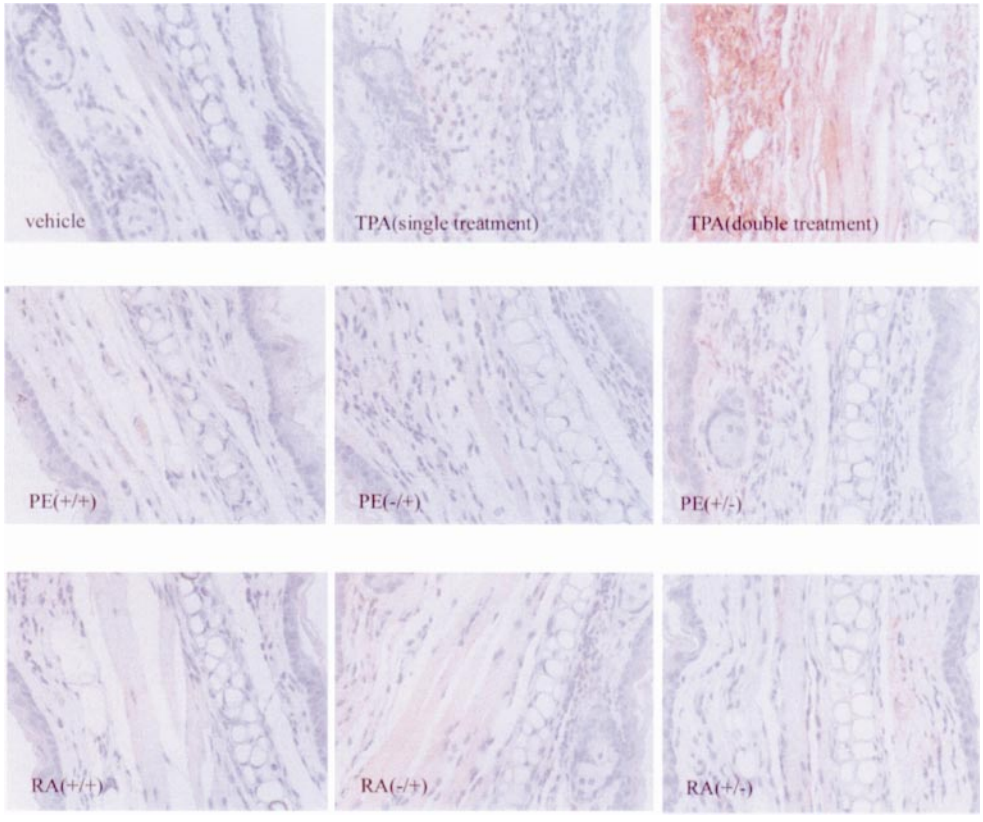


**Fig. 11.** Detection of 8OH-dG adduct in ears of mice treated with TPA. 8OH-dG appears as light brown staining. Vehicle (TPA untreated and RA/PE untreated), TPA single challenged and untreated (TPA single treatment), TPA double challenged and untreated (TPA double treatment), TPA double challenged and double treated (+/+), TPA double challenged and RA/PE treated prior to first TPA treatment (+/−), TPA double challenged and RA/PE treated prior to the second TPA (−/+) treatment with 2.0 mg PE/mouse or 1.35 mg RA/mouse. The second TPA treatment was performed 24 h after the first TPA treatment. Animals were killed under anesthesia 1 h after the second TPA application.

and PE treatment groups. Consistent ith this finding, Nakamura *et al*. (67) reported that RA shows potent superoxide scavenging activity. We confirmed this ability using electron spin resonance *in vitro* (data not shown). In addition, we have shown recently that RA has antioxidative activity *in vivo* in an LPS- and D-galactosamine-induced liver injury model (16).

We conclude that RA plays an anticarcinogenic role in *P.frutescens* extract by two independent effects: anti-inflammatory activities such as inhibition of adhesion molecule, chemokine and eicosanoid synthesis, and antioxidative activity such as inhibition of oxidative DNA injury.

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*Conflict of interest statement*

N.Osakabe is employed by Meiji Seika Kaisha Ltd, the manufacturers of foods and medicines, and is currently conducting research sponsored by this company.

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