



# Inhibitory effects of the ginsenoside Rg<sub>3</sub> on phorbol ester-induced cyclooxygenase-2 expression, NF-κB activation and tumor promotion<sup>☆</sup>

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## Abstract

Our previous studies demonstrated the anti-oxidant and anti-tumor promotional properties of the methanol extract of heat-processed *Panax ginseng* C.A. Meyer [Cancer Lett. 150 (2000) 41]. In the present work, we have evaluated anti-inflammatory as well as anti-tumor promoting effects of Rg<sub>3</sub>, a major ginsenoside derived from heat-processed ginseng. Pretreatment of dorsal skins of female ICR mice with Rg<sub>3</sub> significantly inhibited 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced ornithine decarboxylase activity and 7,12-dimethylbenz[*a*]anthracene-initiated papilloma formation. In another experiment, Rg<sub>3</sub> pretreatment abrogated the expression of cyclooxygenase-2 in TPA-stimulated mouse skin. Rg<sub>3</sub> also inhibited the TPA-induced activation of the eukaryotic transcription factor, NF-κB in both mouse skin and cultured human pro-myelocytic leukemia (HL-60) cells. Moreover, Rg<sub>3</sub> exerted potent inhibitory effects on the activation of another transcription factor, activator protein-1 (AP-1) that is responsible for *c-jun* and *c-fos* oncogenic transactivation. Based on these findings, it is likely that the anti-tumor promoting activity of Rg<sub>3</sub> is mediated possibly through down-regulation of NF-κB and AP-1 transcription factors. © 2002 Elsevier Science B.V. All rights reserved.

**Keywords:** Anti-tumor promotion; AP-1; Cyclooxygenase-2; Ginseng; Ginsenoside Rg<sub>3</sub>; HL-60 cells; Mouse skin carcinogenesis; NF-κB

## 1. Introduction

A relatively large number of anti-oxidative and anti-inflammatory substances derived from edible plants have been shown to possess substantial chemopreventive properties [1,2]. For instance, tea polyphenols

such as epigallocatechin gallate, resveratrol present in red wine, and curcumin in turmeric possess potent anti-carcinogenic activities (reviewed in [1] and references therein). The role of these phytochemicals in dietary cancer prevention has been extensively investigated and well-documented [1,2]. The roots or rhizome of several varieties of *Panax* plants including *P. ginseng*, *P. notoginseng*, *P. japonicus* and *P. quinquefolium* have been used in traditional oriental medicine for the treatment of many disorders [3]. Among these *Panax* geni, *P. ginseng* C.A. Meyer is one of the most widely used medicinal plants throughout Far East Asian countries including China, Korea

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and Japan. According to an epidemiologic study conducted in Korea, chronic intake of ginseng has been associated with a decreased incidence of cancers such as esophageal, gastric, colorectal, and pulmonary tumors [4–6]. Certain fractions or purified ingredients of ginseng have been shown to exert anti-tumorigenic or anti-mutagenic activities in experimental animals and cultured cells [5–15]. However, the exact molecular mechanisms that underly chemopreventive effects of ginseng remain largely unknown. In multistage carcinogenesis, tumor promotion is closely related to such biochemical and molecular events as increased activity and/or expression of ornithine decarboxylase (ODC), generation of reactive oxygen species (e.g. superoxide anion, hydrogen peroxide, etc.), acute inflammation characterized by skin edema, hyperplasia, increased expression/release of pro-inflammatory cytokines (e.g. TNF- $\alpha$  and interleukin-1 $\alpha$ ) and aberrant up-regulation of pro-inflammatory enzymes including cyclooxygenase-2 (COX-2). Our previous studies have demonstrated that the methanol extract of the heat-processed *P. ginseng* C.A. Meyer is capable of inhibiting lipid peroxidation in rat brain homogenates and also scavenging superoxide anion generated by xanthine/xanthine oxidase or 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in cultured human pro-myelocytic leukemia (HL-60) cells [9]. Topical application of the same extract onto dorsal skins of mice also attenuated TPA-induced ODC activity and tumor promotion [9]. In the present study, we have investigated the effects of Rg<sub>3</sub> (structure shown in Fig. 1), a major ginsenoside derived from the heat-processed ginseng (Sun Ginseng), on TPA-induced mouse skin tumor promotion. In an attempt to elucidate the biochemical mechanisms responsible for the possible anti-tumor promoting activity of Rg<sub>3</sub>, we have examined its effects on the activity of ODC, expression of COX-2 and the acti-

vation of the transcription factor NF- $\kappa$ B and activator protein-1 (AP-1) in TPA-stimulated mouse skin.

## 2. Materials and methods

### 2.1. Materials

Rg<sub>3</sub> was prepared and purified as described previously [16]. RPMI 1640, fetal bovine serum, [ $\gamma$ -<sup>32</sup>P]ATP and the electrophoretic mobility shift assay (EMSA) kit were obtained from Gibco BRL Life Science (Grand Island, NY, USA). TPA was purchased from Alexis Biochemicals Co. (San Diego, CA, USA). Protease inhibitor cocktail tablets were supplied from Roche Molecular Biochemicals (Mannheim, Germany). Goat COX-2 polyclonal antibody was the product of Santa Cruz (Santa Cruz, CA, USA). The anti-goat horseradish peroxidase conjugated-secondary antibody was purchased from Zymed Laboratories (San Francisco, CA, USA). L-[1-<sup>14</sup>C]Ornithine hydrochloride was purchased from ICN Chemicals (Costa Mesa, CA, USA). Oligonucleotides containing NF- $\kappa$ B and AP-1 consensus sequences were obtained from Promega (Madison, WI, USA). Female ICR mice were obtained from Joong-Ang Experimental Animal Co. (Taejon, Korea) and maintained under standard conditions. The mice were shaved with an electric clipper, and hair depilatory cream was applied 2 days prior to the experiments.

### 2.2. Determination of mouse epidermal ODC activity

The female ICR mice were topically treated on their shaven backs with various amounts of Rg<sub>3</sub> 30 min prior to topical application of 10 nmol TPA and killed by cervical dislocation 5 h later. Skin was excised, and the fat was removed by razor. The fat-free mouse epidermis was homogenized in 50 mM sodium phosphate buffer (pH 7.2) containing 0.1 mM pyridoxal phosphate and 0.1 mM EDTA and centrifuged at 15,000  $\times$  g for 20 min. The ODC activity in the soluble fraction was determined using L-[1-<sup>14</sup>C]ornithine hydrochloride as a substrate [9]. After incubation at 37 °C for 1 h, the release of <sup>14</sup>CO<sub>2</sub> was measured by liquid scintillation counting. The protein concentration was determined by using the BCA protein assay kit (Pierce product, Rockford, IL, USA).

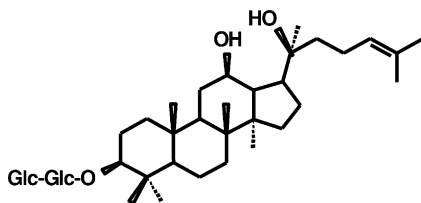


Fig. 1. The chemical structure of Rg<sub>3</sub>.

### 2.3. Two-stage mouse skin tumor experiment

Groups of 25–30 female ICR mice of 6–7 weeks of age were treated in their shaven backs with a single topical application of 7,12-dimethylbenz(a)anthracene (DMBA; 0.2  $\mu\text{mol}$ ) in 0.2 ml acetone or solvent alone. Starting 1 week after the initiation, 7.5 nmol TPA was topically applied thrice a week until termination of the experiment according to the protocol reported elsewhere [17]. Rg<sub>3</sub> was topically applied 30 min before each TPA treatment.

### 2.4. Western blot analysis of mouse epidermal COX-2

The female ICR mice were topically treated on their shaven backs with indicated doses of Rg<sub>3</sub> 30 min before TPA treatment and killed by cervical dislocation 5 h later. Total protein was isolated and quantified. Collected tissues were lysed in 120  $\mu\text{l}$  of ice-cold lysis buffer (150 mM NaCl, 0.5% Triton-X 100, 50 mM Tris-HCl, pH 7.4, 20 mM EGTA, 1 mM DTT, 1 mM Na<sub>3</sub>VO<sub>4</sub> and protease inhibitor cocktail tablet) for 40 min. Lysates were centrifuged at 14,800  $\times g$  for 30 min, and aliquots of supernatant containing 30  $\mu\text{g}$  protein were boiled in SDS sample buffer for 5 min before electrophoresis on 12% SDS-polyacrylamide gel. After a 3 h transfer of SDS-polyacrylamide gel to PVDF membrane, the blots were blocked with 5% fat-free dry milk-PBST buffer (phosphate-buffered saline containing 0.1% Tween 20) for 2 h at room temperature and then washed in PBST buffer. The membranes were incubated for 1 h at room temperature with 1:1000 dilution of goat COX-2 polyclonal antibody for 2 h. Blots were rinsed with PBST, then incubated with 1:5000 dilution of anti-goat horseradish peroxidase conjugated-secondary antibody for 1 h and again washed in PBST buffer for 5 min three times [17]. The transferred proteins were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia) according to the manufacturer's procedures.

### 2.5. Preparation of nuclear extracts from mouse skin and HL-60 cells

The mouse skin nuclear extract was prepared as described previously [17,18]. In brief, scraped dorsal

skin of mice was homogenized in 1 ml of hypotonic buffer A [10 mM HEPES, pH 7.8, 10 mM KCl, 2 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride (PMSF)]. To the homogenates was added 125  $\mu\text{l}$  of 10% Nonidet P-40 (NP-40) solution, and the mixture was then centrifuged for 30 s at 14,000  $\times g$ . The nuclei were washed once with 400  $\mu\text{l}$  of buffer A plus 25  $\mu\text{l}$  of 10% NP-40, centrifuged, re-suspended in 50  $\mu\text{l}$  of 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, 10% glycerol) and centrifuged for 5 min at 14,800  $\times g$ . The supernatant containing nuclear proteins was collected and stored at  $-70^\circ\text{C}$  after determination of protein concentrations.

HL-60 cells ( $1 \times 10^6 \text{ ml}^{-1}$ ) were grown in suspension culture in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). For the preparation of nuclear extracts, cells were washed with ice-cold phosphate-buffered saline and suspended in 0.4 ml of lysis buffer (10 mM HEPES, pH 7.9, 0.2 mM PMSF, 0.5 mM DTT, 1.5 mM MgCl<sub>2</sub>, 10 mM KCl). The cells were allowed to swell on ice for 10 min and then centrifuged for 30 s at 14,800  $\times g$ . The nuclear pellet was suspended in 100  $\mu\text{l}$  of ice-cold nuclear extraction buffer [20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM MgCl<sub>2</sub>, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 20% glycerol] which was kept in an ice bath for 20 min with intermittent flicking. The mixture was then centrifuged for 30 s at 14,800  $\times g$ , and the supernatant was either used immediately or stored at  $-70^\circ\text{C}$  for later use.

### 2.6. Electrophoretic mobility shift assay (EMSA)

EMSA for NF- $\kappa$ B DNA binding was performed using a DNA-protein binding detection kit, according to the manufacturer's protocol (GIBCO BRL, Grand Island, NY, USA) as described previously [17–19]. Briefly, the NF- $\kappa$ B oligonucleotide probe (5'-AGT TGA GGG GAC TTT CCC AGG C-3') was labeled with [ $\gamma$ -<sup>32</sup>P]ATP by T4 polynucleotide kinase and purified on a Nick column (Amersham Pharmacia Biotech, Buckinghamshire, UK). The binding reaction was carried out in 25  $\mu\text{l}$  of the mixture containing 5  $\mu\text{l}$  of incubation buffer [10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% glycerol, and 0.1 mg/ml sonicated salmon sperm DNA], 10  $\mu\text{l}$  of nuclear extracts, and 100,000 cpm of [ $\gamma$ -<sup>32</sup>P]-end

labeled oligonucleotides. After 50 min incubation at room temperature, 2  $\mu$ l of 0.1% bromophenol blue was added, and samples were electrophoresed through 6% non-denaturing polyacrylamide gel at 150 V in a cold room for 2 h. Finally, the gel was dried and exposed to X-ray film. Similarly, the AP-1 DNA binding activity was assessed using [ $\gamma$ - $^{32}$ P]-labeled double-strand oligonucleotides (5'-CGC TTC ATG AGT CAG CCG GAA-3') containing the AP-1 binding site.

### 3. Results

ODC is a rate-determining enzyme in biosynthesis of polyamines that play pivotal roles in cell proliferation. Constitutive activation of polyamine biosynthesis by ODC has been implicated in carcinogenesis [20]. Both catalytic activity and protein levels of ODC have been often elevated in transformed cells [21–23]. ODC has been recognized as a biochemical hallmark of tumor promotion [24,25]. Topical application of the dorsal skins of mice with Rg<sub>3</sub> 30 min prior to the application of 10 nmol TPA significantly reduced the ODC activity (Fig. 2). In another experiment, Rg<sub>3</sub> pretreatment markedly suppressed the TPA-induced skin tumor promotion in mouse skin (Fig. 3). Aberrant or inappropriate overexpression of COX-2 has also been implicated in the development of cancer,

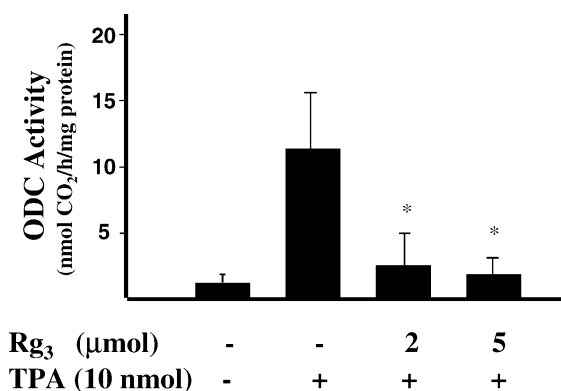


Fig. 2. The inhibitory effect of Rg<sub>3</sub> on TPA-induced ODC activity in mouse skin. Female ICR mice were treated topically with 0  $\mu$ mol, 2 or 5  $\mu$ mol of Rg<sub>3</sub> 0.5 h prior to 10 nmol TPA. Epidermal ODC activity was measured at 5 h later. Control animals were treated with acetone alone. Data are expressed as the means  $\pm$  S.D. from four mice per group. \*Significantly different from the value observed in animals treated with TPA alone.

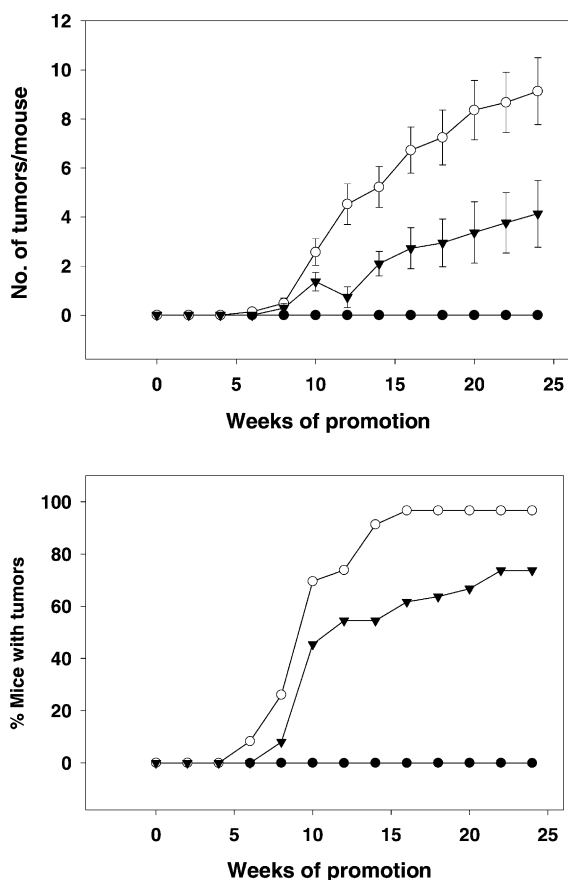


Fig. 3. Inhibition of mouse skin tumor promotion by Rg<sub>3</sub> pretreatment. Groups of 25–30 young female ICR mice were treated with 0  $\mu$ mol (open circle) or 0.2  $\mu$ mol (closed triangle) of Rg<sub>3</sub> 30 min prior to each topical application of TPA after initiation with DMBA. Control animals received topical application of acetone alone (closed circle). Tumor multiplicity data are expressed as means  $\pm$  S.E.

particularly in the stage of promotion or progression, and selective inhibition of COX-2 is hence recognized as one of the most promising and practical strategies for preventing or treating cancer as well as ameliorating inflammatory disorders [26–28]. To test whether Rg<sub>3</sub> could inhibit TPA-induced expression of this pro-inflammatory enzyme, we examined the levels of COX-2 protein in TPA-stimulated mouse skin with and without Rg<sub>3</sub> pretreatment. As illustrated in Fig. 4, topical application of 2 or 5  $\mu$ mol Rg<sub>3</sub> prior to TPA treatment lowered the induction of COX-2 in mouse skin. In an attempt to elucidate the molecular

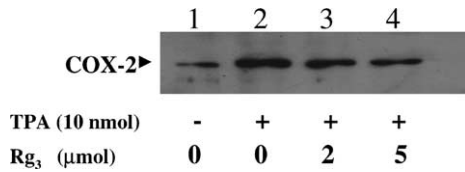


Fig. 4. Effects of Rg<sub>3</sub> on TPA-induced COX-2 expression. Dorsal skins of mice were treated with acetone (lane 1) or 10 nmol TPA in the absence (lane 2) or presence of 2 μmol (lane 3) or 5 μmol (lane 4) of Rg<sub>3</sub>. Mice were killed 5 h after the TPA application for immunoblot analysis of COX-2 as described in Section 2.

mechanisms underlying suppression of TPA-induced expression of COX-2 by Rg<sub>3</sub>, we have examined the effect of this ginsenoside on activation of murine epidermal NF-κB and AP-1 that are ubiquitous eukaryotic transcription factors known to be involved in the regulation of COX-2 induction (reviewed in [29], and see references therein). Previously, we have reported that a single topical application of 10 nmol TPA onto mouse skin caused the maximal NF-κB DNA binding at 1 h as determined by the gel shift assay [18]. While 5 or 10 nmol TPA caused marked activation of NF-κB DNA binding at 1 h, much weaker activity was observed at a higher concentration (i.e. 25 nmol) as shown in Fig. 5A. This may be due to negative feed-back loop controlling IκBα degradation and NF-κB activation. Treatment of mouse skin with Rg<sub>3</sub> before or together with a single topical dose of TPA resulted in substantial inhibition of NF-κB activation, whereas the post-treatment exhibited much weaker effects (Fig. 5B). Curcumin, a yellow colored anti-inflammatory ingredient present in the rhizome of turmeric (*Curcuma longa* L, Zingiberaceae), was shown to directly interfere with binding of activated NF-κB to the DNA [19,30]. To determine whether or not Rg<sub>3</sub> could abrogate the DNA binding capability of NF-κB, the nuclear extracts from mouse epidermis stimulated with TPA were incubated in vitro with Rg<sub>3</sub> at room temperature for 1 h, and EMSA was conducted. Unlike curcumin, Rg<sub>3</sub> failed to nullify the ability of pre-activated NF-κB to bind to DNA (Fig. 6A). Similarly, Rg<sub>3</sub> could not block the direct binding of pre-activated AP-1 to DNA (Fig. 6B). The effect of Rg<sub>3</sub> on TPA-induced NF-κB activation was also investigated in cultured cells. HL-60 cells treated with 10 nM TPA resulted in the activation of NF-κB. The activation occurred in as early as 1 h,

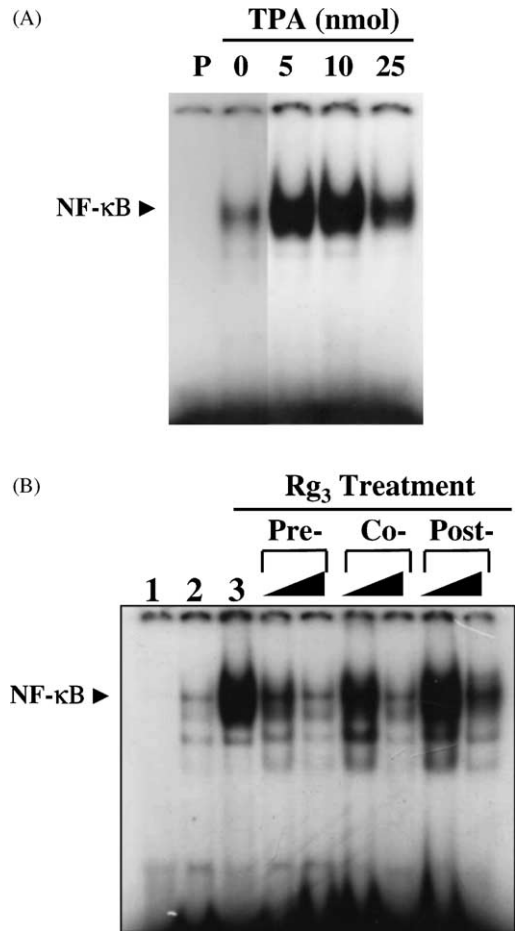


Fig. 5. Induction of TPA-induced NF-κB-DNA binding in mouse skin (A) and its modulation by Rg<sub>3</sub> (B). (A) Female ICR mice were treated with varying doses of TPA for 1 h, and epidermal nuclear extracts were prepared for EMSA as described in Section 2. Control animals were treated with acetone in lieu of TPA. ‘P’ denotes probe only. (B) Topical doses (0.5 or 2 μmol) of Rg<sub>3</sub> were topically applied onto dorsal skins of mice 30 min prior, simultaneously with, or 30 min after 10 nmol TPA. Mice were killed 1 h later for preparation of epidermal nuclear extracts. Lane 1, probe only; lane 2, acetone control; lane 3, TPA control.

sustained up to 8 h, and was concentration-dependent [19]. The specificity and identity of NF-κB activated in TPA-stimulated HL-60 cells were verified by the competition and the supershift assays, respectively [19]. HL-60 cells, when pretreated with Rg<sub>3</sub>, exhibited concentration-dependent inhibition of TPA-induced NF-κB activation, while Rg<sub>3</sub> alone did not affect the constitutive DNA binding activity of NF-κB (Fig. 7).

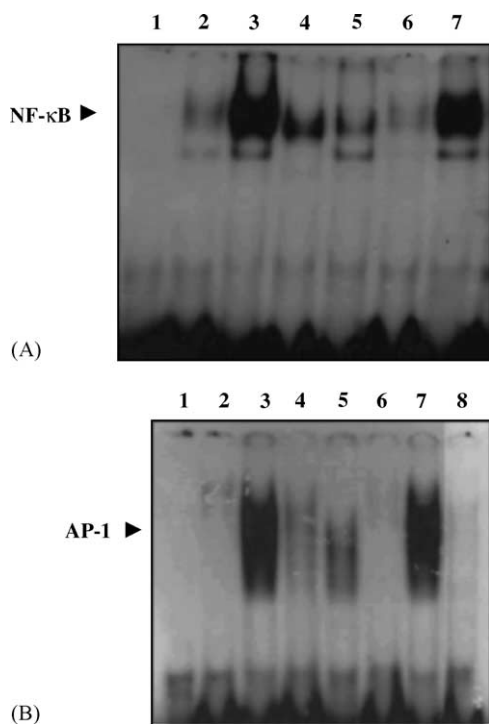


Fig. 6. Comparison of effects of curcumin and Rg<sub>3</sub> on TPA-induced DNA binding of NF-κB (A) or AP-1 (B) in mouse skin. Dorsal skins of female ICR mice were treated topically with acetone (lane 2) or 10 nmol TPA (lane 3). For some animals, 1 μmol curcumin (lane 4) or 0.5 μmol Rg<sub>3</sub> (lane 5) was topically applied 30 min prior to TPA. Animals were killed 1 h later, and epidermal nuclear extracts were prepared for EMSA. For lanes 5 and 7, the nuclear extracts from TPA-stimulated epidermis were mixed *in vitro* with curcumin (1 μmol) or Rg<sub>3</sub> (0.5 μmol), respectively in a final volume of 27 μl at room temperature for 20 min and subjected to EMSA for determining NF-κB DNA binding. Lane 1 represents the probe only. Lane 8 represents competition of AP-1 DNA binding by addition of excess amounts of the unlabeled AP-1 oligonucleotide probe.

#### 4. Discussion

Since oxidative stress and inflammation are closely linked to carcinogenesis, it is conceivable that ginseng that possesses anti-oxidative [9,10,31] and anti-inflammatory [9,32] activities can exert chemopreventive activities. Crude extracts of ginseng as well as its saponin constituents have been tested for their cancer chemopreventive and chemoprotective activities [11–15]. The methanolic extract of red ginseng enhanced the uptake of mitomycin C by the

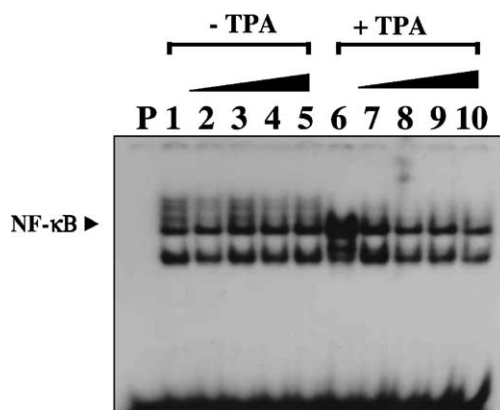


Fig. 7. Effects of Rg<sub>3</sub> on TPA-induced activation of NF-κB in HL-60 cells. The cells were treated with DMSO alone or TPA (10 nM) with and without pretreatment for 30 min of 1, 10, 50 or 100 μM Rg<sub>3</sub>. Cells were harvested 1 h later, and nuclear extracts were prepared for determining the sequence specific NF-κB DNA binding activity by using the [ $\gamma$ -<sup>32</sup>P]-labeled oligonucleotides. 'P', probe only.

tumor cells and potentiated its cytotoxic effect [33]. In another study, cytotoxicity of mitomycin C towards tumor cells in culture and B16 melanoma inoculated mice was potentiated by panaxytriol [34]. Moreover, the anti-metastatic potential of ginseng saponins such as Rg<sub>3</sub> and Rb<sub>2</sub>, has been demonstrated [35–38].

ODC is the rate-limiting enzyme that mediates the biosynthesis of polyamines by converting L-ornithine into putrescine. ODC has particularly been considered as a biochemical hallmark of tumor promotion that is related to hyperproliferation of initiated cells. Studies in mouse skin [25] and other organs [39,40] have shown intimate correlation between the induction of the ODC activity and the tumor promoting ability of a variety of substances. Moreover, ODC overexpression apparently stimulates anchorage-independent growth of human breast epithelial (MCF-10A) cells in a soft agar assay [41], through cooperation with HER-2/neu oncogene [42,43] which might be regulated by mitogen-activated protein kinase (MAPK) signaling cascades involving extracellular signal-regulated protein kinase (ERK). Conversely, tumor formation can be prevented by the agents capable of blocking ODC induction, such as retinoids [44], dimethylfluorornithine [45], or indomethacin [46]. ODC inhibitors are hence considered as potential chemopreventive agents [47]. Based on its significant inhibitory

effect on TPA-induced mouse epidermal ODC activity, it is likely that Rg<sub>3</sub> exerts the chemopreventive effects by inhibiting TPA-induced mouse epidermal proliferation, thereby attenuating promotion of papillomagenesis.

Besides ODC, COX-2 has also been considered to be an important enzyme that plays a role in the pathogenesis of cancer, and becomes a potential target for cancer chemoprevention [48] and therapy [26,28]. An expanding body of evidence indicates that selective inhibition of COX-2 is one of the promising approaches to prevent cancer [48]. Thus, down-regulation of COX-2, together with inhibition of ODC activity, by Rg<sub>3</sub> is likely to contribute to its anti-tumor promoting effects in mouse skin.

There is compelling evidence that expression of COX-2 is mainly regulated at the transcriptional level [49]. The promoter region of COX-2 contains at least one putative NF- $\kappa$ B consensus sequence that acts as a positive regulatory element for COX-2 transcription [50]. Results from numerous investigations support the notion that NF- $\kappa$ B is responsible for the regulation of COX-2 expression (reviewed in [29]). Recent studies from this laboratory have revealed that TPA-induced COX-2 expression in mouse skin is significantly reduced by topical application of the NF- $\kappa$ B inhibitor pyrrolidine dithiocarbamate [29], suggestive of a functional role of this transcription factor in regulating COX-2 induction in TPA-stimulated murine epidermis.

Since Rg<sub>3</sub> inhibited TPA-induced COX-2 expression in mouse skin at doses that also hampered the NF- $\kappa$ B DNA binding, it is conceivable that the suppression of COX-2 by this ginsenoside might be achieved, at least in part, via the NF- $\kappa$ B pathway. NF- $\kappa$ B is a ubiquitous eukaryotic transcription factor that is a dimer of Rel family proteins. This transcription factor is usually sequestered in the cytoplasm as an inactive complex with inhibitory kappa B (I $\kappa$ B). However, upon exposure of cells to extracellular stimuli from a variety of pathogens or stress-inducing agents, I $\kappa$ B gets functionally activated, and NF- $\kappa$ B can subsequently translocate into nucleus, triggering transcription of a wide array of genes many of which encode proteins crucial for diverse physiological responses. Budunova et al. [51] have reported that constitutive activation of NF- $\kappa$ B is critical in mouse skin carcinogenesis. Moreover, selective in-

hibition of NF- $\kappa$ B signaling in murine skin by targeted overexpression of a super-repressor form of I $\kappa$ B $\alpha$ , augmented spontaneous development of squamous cell carcinoma [52], and functional blockade of NF- $\kappa$ B by expressing dominant-negative I $\kappa$ B mutant in transgenic mouse or in cultured primary human keratinocytes which were subsequently transplanted into mouse skin produced hyperplastic epithelium [53]. More recently, endogenous redox stress resulting from reactive oxygen species spontaneously produced by NAD(P)H:quinone oxidoreductase activity has been shown to contribute to constitutive activation of NF- $\kappa$ B in certain types of human melanoma cell lines, which accounts for hyperproliferative properties of these cells [54]. Similar constitutive activation of this ubiquitous eukaryotic transcription factor has been found in malignant tumors or transformed cells of other origin [55–60]. Certain edible chemopreventive phytochemicals including curcumin [2,19,61], caffeic acid [62], capsaicin [3,18,63], and epigallocatechin 3-gallate [64] have been shown to inhibit NF- $\kappa$ B activation through blockade of I $\kappa$ B degradation. It would be of interest to test whether Rg<sub>3</sub> has similar inhibitory effects on the degradation of I $\kappa$ B, thereby inhibiting translocation of NF- $\kappa$ B to nucleus for DNA binding.

The intracellular signaling pathways mediating ODC induction is not yet well-elucidated. ODC induction appears to be regulated by p44/42 MAPK (ERK1/2), but not p38 MAPK based on the relative effects of specific inhibitors of both MAPKs in cultured human ECV304 cells [65] and also in L1210 leukemia cells [66]. p38 MAPK, on the contrary, appears to down-regulates ODC activity. Thus, interleukin-1-induced suppression of growth and ODC activity of human melanoma cell line (A375-C2-1) was reversed by addition of the selective inhibitor of p38 MAPK, SB203580 [67]. It has been suggested that NF- $\kappa$ B, in cooperation with ERK, plays a functional role in growth stimulation and ODC induction by TNF- $\alpha$  or lipopolysaccharide in cultured chick embryo cardiomyocytes [68]. Blockade of polyamine synthesis by the ODC inhibitor,  $\alpha$ -difluoromethylornithine resulted in activation of NF- $\kappa$ B in the rat intestinal mucosal IEC-6 cell line through degradation of I $\kappa$ B $\alpha$  [69]. By using mouse epidermal JB6 cells expressing a novel transformation suppressor pdcd4, Yang et al. [70] have tested the possible involvement of NF- $\kappa$ B or AP-1 in transcription of ODC. While AP-1-dependent

transcriptional activity was inhibited by *pdcd4* expression, NF- $\kappa$ B-dependent transcription was slightly increased without alteration of ODC activity. Although ODC activity was suppressed in parallel with NF- $\kappa$ B inactivation by Rg<sub>3</sub> pretreatment in mouse skin, it remains unclear whether these two events are causally linked. The use of genetically tractable mouse models, especially those in which the gene encoding one of the functional subunits of NF- $\kappa$ B is mutated or deleted, will probably help to better understand the role of NF- $\kappa$ B in induction of ODC expression in mouse skin. Transcription factors other than NF- $\kappa$ B and AP-1 have recently been reported to be responsible for transcriptional regulation of ODC gene [71,72], and it will be worthwhile to examine whether Rg<sub>3</sub> targets these transcription factors in the context of its anti-tumor promoting activity.

NF- $\kappa$ B has been recognized as a redox-sensitive transcription factor [73]. Schreck et al. [74] have proposed an inter-relationship between redox status and NF- $\kappa$ B activation processes, focusing the role of reactive oxygen species as a potential mediator of NF- $\kappa$ B signaling cascades. Since phorbol ester is known to produce reactive oxygen species, the suppression of TPA-induced activation of NF- $\kappa$ B by Rg<sub>3</sub> appears to be attributable to its anti-oxidant activity. NF- $\kappa$ B not only modulates gene expression but also cooperates with AP-1 to promote proliferation and transformation [75]. Like NF- $\kappa$ B, AP-1 is a redox-sensitive transcription factor, which is a complex of oncogene protein of c-Jun and c-Fos families. *c-jun* and *c-fos* are induced by a broad range of extracellular stimuli including UVB, UVC, TPA, growth factors and transforming oncogenes, and their protein products function as intermediate transcriptional regulators in signal cascades leading to cell proliferation and transformation [76]. Close association has been observed between AP-1 activation and tumor promotion. Thus, skin tumors with increased levels of AP-1 effector gene expression displayed the high degree of malignancy [77]. AP-1 was also found to be required for tumor promoter-induced cell transformation in vitro [78] and tumor promotion in an animal model [79,80]. Therefore, suppression of AP-1 DNA binding by Rg<sub>3</sub> is likely to contribute to its anti-tumor promoting activity in mouse skin carcinogenesis. Identification of upstream molecules that Rg<sub>3</sub> targets in the context of inactivation of NF- $\kappa$ B and AP-1 merits further investigation.

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