Carcinogenesis vol.26 no.2 pp.359–367, 2005
doi:10.1093/carcin/bgh313

Antitumor promotional effects of a novel intestinal bacterial metabolite (IH-901) derived from the protopanaxadiol-type ginsenosides in mouse skin

Ji-Yoon Lee[1], Jun-Wan Shin[1,*], Kyung-Soo Chun[1,*], Kwang-Kyun Park[2], Won-Yoon Chung[2], Yung-Jue Bang[3], Jong-Hwan Sung[4] and Young-Joon Surh[1,5]

[1]National Research Laboratory of Molecular Carcinogenesis and Chemoprevention, College of Pharmacy, Seoul National University, Seoul 151-742, Korea, [2]College of Dentistry, Yonsei University, Seoul 120-742, [3]Cancer Research Institute, Seoul National University College of Medicine, Seoul 110-799 and [4]Ilhwa Co., Ltd, Guri-shi, Gyeonggi-do 471-711, South Korea

[5]To whom correspondence should be addressed
Email: surh@plaza.snu.ac.kr

Epidemiological studies have demonstrated that ginseng intake decreases the risk of cancer. Ginseng saponins (ginsenosides) have been regarded as principal components responsible for the majority of pharmacological activities exerted by ginseng. IH-901 [20-*O*-β-D-glucopyranosyl-20(S)-protopanaxadiol], an intestinal bacterial metabolite derived from protopanaxadiol-type saponins of *Panax ginseng* C.A. Meyer, has been reported to possess antitumor effects, including inhibition of invasion, metastasis and angiogenesis and induction of tumor cell apoptosis. Tumor promotion often accompanies an elevated ornithine decarboxylase (ODC) activity, acute inflammation and induction of cyclooxygenase-2 (COX-2) activity. Here we examined the effects of IH-901 on tumor promotion and related molecular events in mouse skin *in vivo*. Mouse ear edema induced by the prototype tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was repressed by IH-901 pre-treatment in a dose-dependent manner. Topical application of IH-901 onto shaven backs of female ICR mice led to the inhibition of TPA-induced expression of COX-2 and production of prostaglandin E_2. The eukaryotic transcription factor NF-κB has been involved in intracellular signaling pathways associated with inflammation and carcinogenesis. IH-901 pre-treatment inhibited TPA-induced epidermal NF-κB DNA binding in mouse skin, which appeared to be mediated by blocking phosphorylation and subsequent degradation of IκBα. In an attempt to elucidate the molecular mechanisms by which IH-901 inactivates NF-κB, its effects on activation of upstream signaling kinases were explored. IH-901 also inhibited the activation of ERK1/2 and Akt signaling. When IH-901 was treated topically prior to TPA, expression and activity of ODC were inhibited dose-dependently. In addition, IH-901 given prior to each topical dose of TPA markedly lowered the number of papillomas in mouse skin induced by 7, 12-dimethylbenz[*a*]anthracene. Taken together, these findings suggest that IH-901 exerts anti-inflammatory effects by inhibiting TPA-induced COX-2 expression, which may contribute to its antitumor-promoting effects on mouse skin carcinogenesis.

Introduction

There have been considerable efforts to search for naturally occurring substances for the intervention of carcinogenesis. Many components derived from dietary or medicinal plants have been found to possess substantial chemopreventive properties. The roots or rhizome of several varieties of *Panax* plants (e.g. *Panax ginseng*, *Panax notoginseng*, *Panax japonicus* and *Panax quinquefolium*) have been used in traditional oriental medicine for the treatment of many disorders, such as inflammation and cancer as well as for enhancing physical strength. Among them, *P.ginseng* C. A. Meyer is most widely used in Asia. It has diverse biological and pharmacological effects, which are mainly attributed to triterpenoid saponin components, collectively named ginsenosides (1). A number of saponins isolated so far from ginseng and its congeners have been extensively investigated with regard to their possible antitumor activity.

Recently, several ginseng saponin metabolites formed by intestinal bacteria were identified after oral administration of ginseng extracts in humans and rats. For example, ginsenoside Rh_1 is a metabolite of ginsenoside Rg_1, but both have different effects on the production of cytokines in THP-1 cells stimulated by bacterial lipopolysaccharide and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) (2). Another well-defined metabolite of ginsenosides is IH-901 (also known as M1 or compound K). IH-901 [20-*O*-β-D-glucopyranosyl-20(S)-protopanaxadiol] (structure shown in Figure 1) is one of the metabolites detected in blood after the oral administration of ginsenoside Rb_1, Rb_2 or Rc to mice, and is speculated to be the major form of protopanaxadiol saponins absorbed from the intestine (3–6). IH-901 was found to be rapidly absorbed from the gastrointestinal tract after oral administration. On the other hand, IH-901 appeared late and was retained for a long period of time in the plasma after oral administration of ginsenoside-Rb_1, which itself is hardly absorbed (7). It has been revealed that *Prevotella oris* (8) and *Eubacterium* sp. A-44 (7) are major bacterial species capable of hydrolyzing Rb_1 to IH-901 as illustrated in Figure 1. IH-901 has been reported to inhibit glucose uptake by tumor cells and to reverse the multi-drug resistance in tumor cells (9). It also has inhibitory effects on benzo[*a*]pyrene-induced mutagenicity and clastogenicity (10). IH-901 exerts an anti-metastatic activity partly through inhibition of tumor invasion (11). It also has anti-platelet and anti-angiogenic activities (12), and causes suppression of primary tumor growth in a spontaneous metastasis model (13). Moreover, IH-901 has

Abbreviations: AP-1, activator protein-1; COX-2, cyclooxygenase-2; MAP, mitogen-activated protein; ODC, ornithine decarboxylase; PI3-K, phosphoinositide 3-kinase; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

*These authors contributed equally to this work.

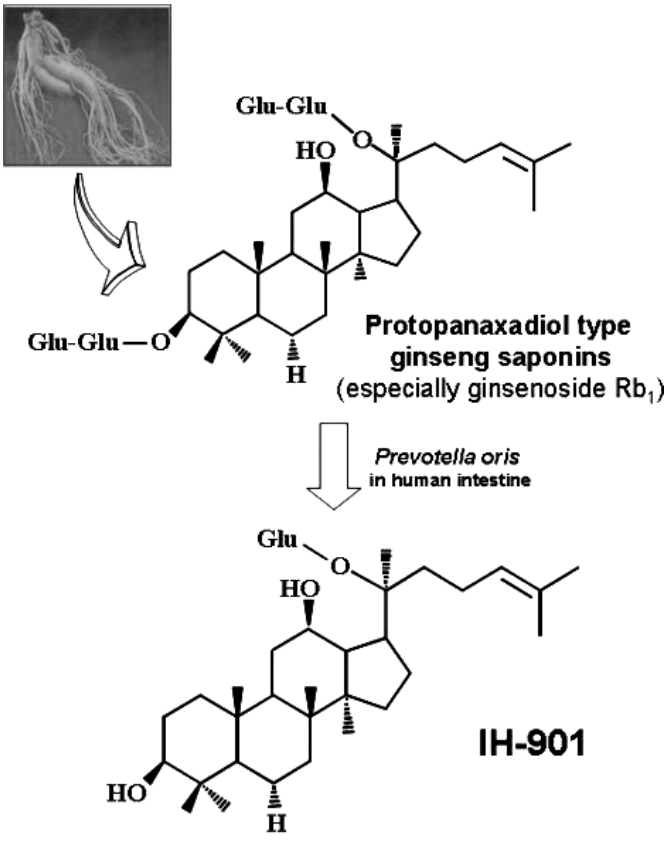

Fig. 1. Metabolic formation of IH-901. IH-901 is formed from protopanaxadiol-type ginsenosides, especially ginsenoside-Rb1, by *P.oris* in the intestine after oral administration of ginseng.

the ability to induce apoptosis in several tumor cell lines (14–18).

Tumor promotion often accompanies an elevated ornithine decarboxylase (ODC) activity or its expression, generation of free radicals and reactive oxygen species, depletion of cellular antioxidant capacity and acute inflammation characterized by skin edema and hyperplasia, and induction of cyclooxygenase-2 (COX-2) (19). As part of our research program to evaluate the chemopreventive potential of IH-901, we have examined the antitumor promotional activity of IH-901 by determining its effects on TPA-induced papillomagenesis as well as expression of COX-2 and ODC. To further elucidate the molecular mechanisms underlying the antitumor-promoting activity of IH-901, its effects on TPA-induced activation of NF-κB and the upstream signaling kinases were also explored.

Materials and methods

Chemicals

IH-901 was prepared by large-scale incubation of protopanaxadiol-type ginseng saponins (5 g) with human intestinal bacteria subcultured in general anaerobic medium at 37°C for 2 days. After incubation, the cultured media were extracted with *n*-BuOH, washed with water and saline successively, and centrifuged. The supernatant was concentrated *in vacuo* and subjected to column chromatography over Kieselgel 60 with $CHCl_3$-MeOH-H_2O (70:30:5T, v/v) and LiChroprep RP-8 using 80–85% MeOH as eluent to afford IH-901 (320 mg), which was characterized by mass spectroscopy and ^{1}H- and ^{13}C-nuclear magnetic resonance spectrometry as reported previously (4). The purity of the compound as assessed by HPLC was >98%. TPA was obtained from Alexis Biochemicals (San Diego, CA). All other chemicals used were in the purest form available commercially.

Animals

Female ICR mice (6–7 weeks of age) were supplied from the Dae-Han Bio-link Experimental Animal Center (Daejeon, Korea). The animals were housed in climate-controlled quarters (24 ± 1°C at 50% humidity) with a 12-h light/12-h dark cycle. The dorsal side of the skin was shaved using an electric clipper, and only those animals in the resting phase of the hair cycle were used in all experiments.

Measurement of mouse ear edema

Groups of four to six female ICR mice were treated on their right ear with IH-901 in 20 μl 10% H_2O in acetone or the vehicle alone 30 min prior to application of 5 nmol TPA. The mice were killed 5 h later, and ear punches were prepared. The increased weight of the ear punch from TPA-treated mice was a measure of inflammation.

Measurement of PGE_2

The female ICR mice (5 mice/group) were topically treated on their shaven backs with indicated doses of IH-901 30 min prior to TPA (10 nmol) application and were killed by cervical dislocation 5 h later. IH-901 was dissolved in 200 μl 10% H_2O in acetone, and TPA was dissolved in 200 μl of acetone and applied to the dorsal shaven area. The pulverized skin was homogenized with ice-cold ethanol and centrifuged for 10 min at 3000 *g*. Supernatant was diluted to 15% with respect to ethanol by adding 0.1 M sodium formate, and pelleted protein was dissolved in 8 M urea. The diluted supernatant was applied to a pre-activated Amprep^{TM} C-18 reverse phase cartridge (Amersham Pharmacia Biotech, Buckinghamshire, UK), and eicosanoids were released by ethyl-acetate containing 1% methanol. The extract was evaporated to dryness under a gentle stream of nitrogen and re-suspended in an enzyme immunoassay buffer. The amounts of PGE_2 were measured by using the PGE_2 enzyme-immunoassay (EIA) kit (Amersham Pharmacia Biotech Inc.) according to the manufacturer's protocol. The quantity of dissolved protein was determined by the BCA method.

Western blot analysis

For isolation of protein from mouse skin, the dorsal skin was excised, and after the fat was removed on ice, the remaining skin tissues were immediately placed in liquid nitrogen and pulverized in mortar. The pulverized skin was homogenized on ice for 20 s with a Polytron tissue homogenizer and lysed in 2 ml ice-cold lysis buffer [150 mM NaCl, 0.5% Triton X-100, 50 mM Tris–HCl (pH 7.4), 20 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM Na_3VO_4, protease inhibitor cocktail tablet (Roche Molecular Biochemicals, Mannheim, Germany)] for 10 min. Lysates were centrifuged at 12 000 *g* for 20 min, and supernatant containing 30 μg protein was boiled in sodium dodecyl sulfate (SDS) sample loading buffer for 10 min before electrophoresis on 12% SDS–polyacrylamide gel. After electrophoresis for 2 h, proteins in SDS–polyacrylamide gel were transferred to PVDF membrane (Gelman Laboratory, Ann Arbor, MI), and the blots were blocked with 5% non-fat dry milk-PBST buffer [phosphate-buffered saline (PBS) containing 0.1% Tween-20] for 60 min at room temperature. The membranes were incubated for 2 h at room temperature with 1:1000 dilution of the following antibodies: COX-2 and COX-1 (Cayman Chemical, Ann Arbor, MI), p65 (Zymed Laboratories, San Francisco, CA), ERK (Santa Cruz Biotechnology, Santa Cruz, CA), Akt and phospho-Akt polyclonal antibodies (Cell Signaling Technology, Beverly, MA), phospho-ERK (Santa Cruz Biotechnology) and ODC (NeoMarkers, Fremont, CA) monoclonal antibodies. Equal lane loading was assessed using actin (Sigma-Aldrich, St Louis, MO). The blots were rinsed three times with PBST buffer for 5 min each. Washed blots were incubated with 1:5000 dilution of the horseradish peroxide-conjugated secondary antibody (Zymed Laboratories, San Francisco, CA) and then washed again three times with PBST buffer. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Inc.).

Preparation of nuclear extracts

Nuclear extract was prepared from mouse skin as described previously (20). Briefly, scraped dorsal skin of mice was homogenized in 1 ml of ice-cold hypotonic buffer A [10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride (PMSF)]. After a 15-min incubation on ice, the nucleoprotein complexes were lysed with 125 μl of 10% Nonidet P-40 (NP-40) solution, followed by centrifugation for 2 min at 14 800 *g*. The nuclei were washed once with 400 μl of buffer A plus 25 μl of 10% NP-40, centrifuged, re-suspended in 150 μl 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 and 10% glycerol], and centrifuged for 5 min at 14 800 *g*. The supernatant containing nuclear proteins was collected and stored at −70°C after determination of protein concentrations.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed using a DNA–protein biding detection kit (Gibco BRL, Grand Island, NY) according to the manufacturer's protocol. Briefly, the NF-κB oligonucleotide probe (5′-AGT TGA GGG GAC TTT CCC AGG C-3′) was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and purified on a Nick column (Amersham Pharmacia Biotech, Inc.). The binding reaction was carried out in a total volume of 25 μl containing 10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% glycerol, 0.1 mg/ml sonicated salmon sperm DNA, 10 μg of nuclear extracts and 100 000 c.p.m. of the labeled probe. After a 50-min incubation at room temperature, 2 μl of 0.1% bromophenol blue was added, and samples were electrophoresed through a 6% non-denaturating polyacrylamide gel at 150 V in a cold room for 2 h. Finally, the gel was dried and exposed to the X-ray film. EMSA was performed with nuclear extracts from at least three different sets of mouse skin, and the intensity of NF-κB DNA binding was quantified by densitometry.

ODC assay

The female ICR mice were topically treated on their shaven backs with indicated doses of IH-901 30 min before 10 nmol TPA treatment and were killed by cervical dislocation 5 h later. The epidermis was separated by brief heating (55°C for 20 s). The epidermal preparation was homogenized in ice-cold sodium phosphate buffer (50 mM; pH 7.8) containing 0.1 mM pyridoxal phosphate and 0.1 mM EDTA, and centrifuged at 12 000 *g* for 20 min. The ODC activity of the soluble epidermal extracts was determined by measuring the release of $^{14}CO_2$ from DL-[1-^{14}C]ornithine hydrochloride. 100 μl of supernatant was incubated in a final volume of 40 mM sodium phosphate buffer (pH 7.8) containing 8 mM DTT, 0.64 mM pyridoxal phosphate and 0.8 mM EDTA with 2 mM DL-[1-^{14}C]ornithine hydrochloride (sp. act., 2.5 μCi/mmol; Amersham Pharmacia Biotech) as a substrate at 37°C for 1 h. The released $^{14}CO_2$ was captured in filter paper wet in Solvable™ (Du Pont/NEN Research Products, Boston, MA). The amount of radioactivity [^{14}C] was measured by liquid scintillation counting. The protein content was determined by the BCA method. Data are expressed as the means ± SD obtained from 4 to 5 mice/group. *Significantly different from the TPA alone group ($P < 0.005$).

Two-stage mouse skin carcinogenesis

Groups of 25–30 mice were treated on their shaven backs with a single topical application of DMBA (0.2 μmol) in 0.2 ml acetone or the solvent alone. One week after initiation, 15 nmol of TPA in acetone was topically applied twice weekly until termination of the experiment at week 19. Each dose of IH-901 dissolved in 0.2 ml 10% H_2O in acetone was topically applied 30 min before TPA treatment. Beginning 1 week following the promotion, animals were checked on a weekly basis for the presence of papillomas of at least 1 mm in diameter. The results were expressed as the percentage of tumor-bearing mice (incidence) and the average number of tumors per mouse (multiplicity).

Statistical analysis

Data are expressed as means ± SD. Statistical significance of changes was determined by the Student's *t*-test. Differences resulting in *P* values <0.05 were considered to be statistically significant.

Results

Inhibitory effect of IH-901 on TPA-induced mouse ear edema

Since tumor promotion is closely related to inflammation, the anti-inflammatory effect of IH-901 was initially examined. Edema is a typical marker of inflammation. A single topical application of TPA (5 nmol) alone onto the right ears of the mice induced edema. When 0.1, 0.3 and 1 μmol of IH-901 was treated topically 30 min before TPA application, ear edema was suppressed by 17, 57 and 71%, respectively (Figure 2A). IH-901 alone did not induce ear edema.

Inhibitory effects of IH-901 on TPA-induced COX-2 expression and PGE_2 production in mouse skin

We have shown previously that TPA, a prototypic tumor promoter and a mitogen, stimulates COX-2 expression and PGE_2 production in mouse skin (21,22). As illustrated in Figure 2B, IH-901 pre-treatment resulted in marked inhibition of COX-2 expression in TPA-treated mouse skin without altering the COX-1 level. In addition, topical application of the dorsal skin of female ICR mice with 1 μmol IH-901 significantly reduced the TPA-induced PGE_2 synthesis (Figure 2C). IH-901 alone did not affect the constitutive production of PGE_2.

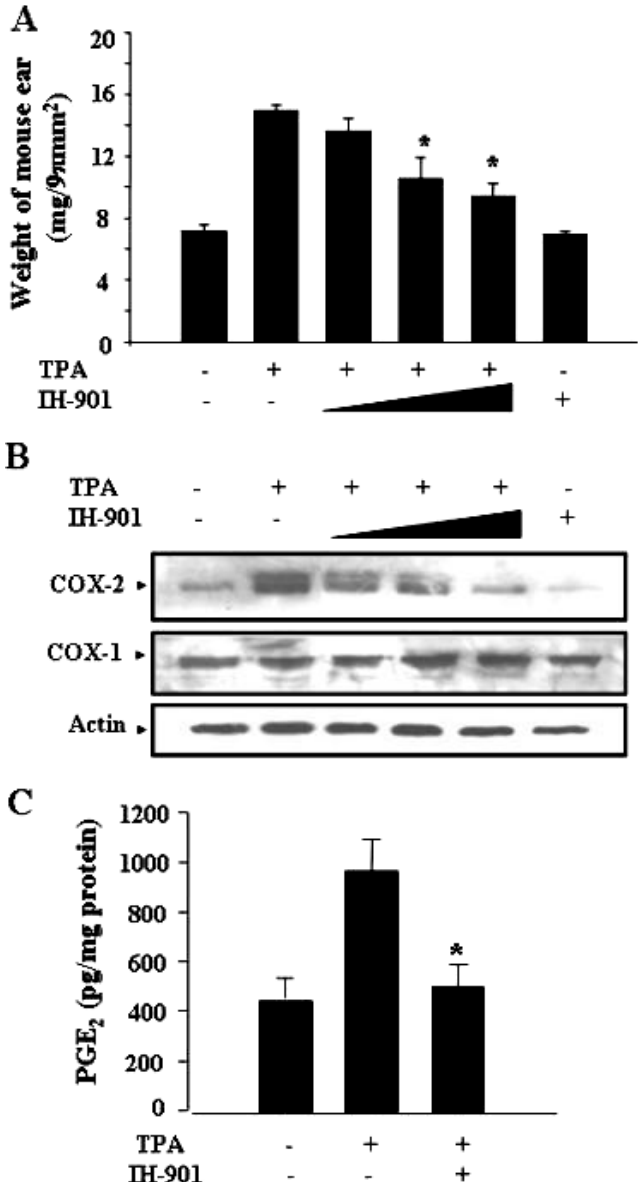

Fig. 2. Effects of IH-901 on TPA-induced mouse ear edema, COX-2 expression and PGE_2 production in mouse skin. (**A**) Female ICR mice were treated on the right ear with IH-901 (0.1, 0.3 or 1 μmol) or vehicle alone 30 min before topical application of TPA (5 nmol) as described in the Materials and Methods. Data are expressed as the means ± SD obtained from 4 to 6 mice/group. *Significantly different from the TPA alone group ($P < 0.005$). (**B**) Female ICR mice were treated topically with 0.2 ml of 10% water in acetone or IH-901 (1, 3 or 10 μmol) in the same volume of vehicle 30 min prior to 10 nmol TPA, and animals were killed 4 h after the TPA treatment. Protein was analyzed for COX-2 by immunoblotting. The immunoblot is representative of three independent experiments. (**C**) Female ICR mice were treated topically with IH-901 (1 μmol) or vehicle alone 30 min prior to 10 nmol TPA, and animals were killed 5 h after the TPA treatment for PGE_2 analysis. Data are expressed as the means ± SD obtained from 5 mice/group. *Significantly different from the group treated with TPA alone ($P < 0.005$).

Inhibitory effect of IH-901 on TPA-induced NF-κB DNA binding

Because NF-κB is known to play a critical role in regulating the expression of COX-2, we have determined whether IH-901

could suppress activation of this transcription factor in mouse skin stimulated with TPA. Our previous studies demonstrated a transient increase in epidermal NF-κB DNA binding after application with 10 nmol TPA, which was abolished by addition of the excess unlabeled probe (20). Effects of IH-901 on NF-κB activation were examined with 1 μmol of the compound topically applied 30 min prior to the TPA treatment. Pre-treatment of IH-901 inhibited TPA-induced NF-κB activation in mouse skin (Figure 3A) while the compound alone did not influence constitutive NF-κB activation (data not shown).

One of the most critical steps in NF-κB activation is the dissociation of IκB, which is mediated through phosphorylation and subsequent proteolytic degradation of this inhibitory subunit. To determine whether the inhibitory effect of IH-901 on NF-κB DNA binding was due to its suppression of IκBα degradation through phosphorylation, the cytoplasmic levels of phosphorylated IκBα were determined by western blot analysis. Topical application of TPA led to substantial increases in the phosphorylation of IκBα, which was significantly repressed by IH-901 pre-treatment (Figure 3B). Likewise, degradation of IκBα was also blocked by IH-901. We also measured the level of p65, the functionally active subunit of NF-κB, in the nucleus. Upon TPA treatment, the nuclear translocation of p65 increased, which was blocked by IH-901. These results indicate that IH-901 inhibits TPA-induced translocation of p65 to the nucleus by blocking IκBα degradation (Figure 3B).

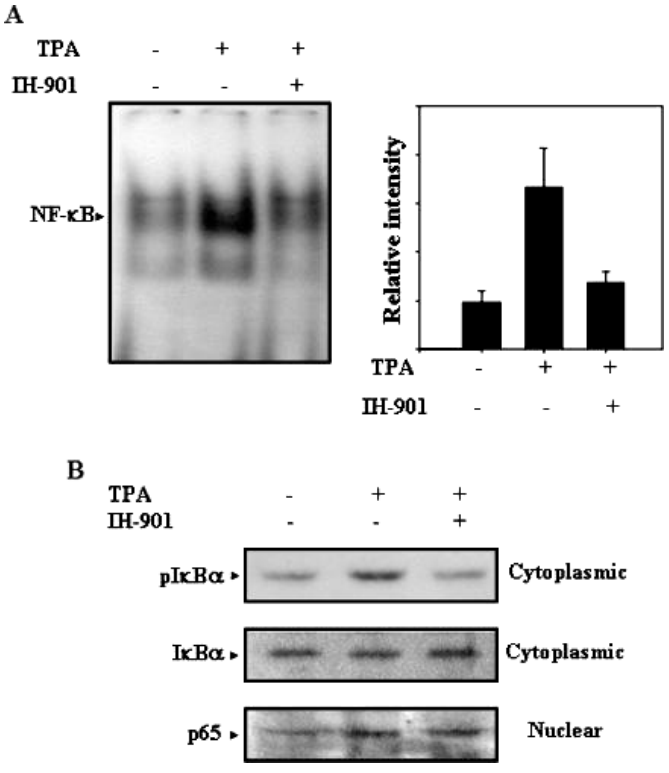

Fig. 3. Effect of IH-901 on TPA-induced NF-κB activation in mouse skin. (**A**) Dorsal skin of mice was treated topically with acetone alone, 10 nmol TPA alone or 1 μmol of IH-901 30 min before TPA treatment. Mice were killed 1 h after the TPA treatment, and epidermal nuclear extracts were prepared and incubated with the radiolabeled oligonucleotides containing the NF-κB consensus sequence for analysis by the EMSA. EMSA was performed with at least three different sets of mouse skin, and the intensity of the NF-κB–DNA complex was quantified. (**B**) Cytoplasmic and nuclear extracts from mouse skin treated 10 nmol TPA for 1 h, with and without IH-901 (1 μmol) pre-treatment, were assayed for phosphorylated IκBα, IκBα and p65 by western blot analysis.

Effect of IH-901 on TPA-induced activation of ERK

Mitogen-activated protein (MAP) kinases are known to regulate NF-κB activation by multiple mechanisms. Accumulating evidence indicates that NF-κB activation is modulated by ERK as well as p38 MAP kinase. We reported previously that U0126, an ultrapotent inhibitor of MAP kinase kinase (MEK)1/2, blocked the NF-κB DNA binding activity, whereas the p38 MAP kinase inhibitor SB203580 failed to (22). This result suggests that the activation of NF-κB occurs via the ERK-dependent pathway in mouse skin. We examined whether IH-901 could down-regulate ERK, thereby inactivating NF-κB and further suppressing the COX-2 induction. IH-901 inhibited the phosphorylation of ERK1/2 in mouse skin (Figure 4A). Under the same experimental conditions, the level of the total form of ERK remained almost constant.

Effect of IH-901 on TPA-induced activation of Akt

Phosphoinositide 3-kinase (PI3-K) and its downstream target Akt/protein kinase B are also known to regulate NF-κB activity (23,24). Akt serves as a stimulator of NF-κB signal pathway by promoting IκB phosphorylation. Figure 4B showed that pre-treatment of IH-901 suppressed TPA-induced phosphorylation of Akt in mouse skin. In order to investigate the possible involvement of Akt in the signaling pathway mediating COX-2 induction, we examined the effect of the PI3-K inhibitor LY294002 on the TPA-induced COX-2 expression. We confirmed that LY294002 at 10 μmol suppressed the activity of PI3-K (Figure 5A). However, LY294002 at a pharmacologically effective dose failed to block the TPA-induced COX-2 expression (Figure 5B). This finding suggests that the PI3-K–Akt down-regulation by IH-901 may not contribute substantially to its inhibition of TPA-induced COX-2 expression in mouse skin.

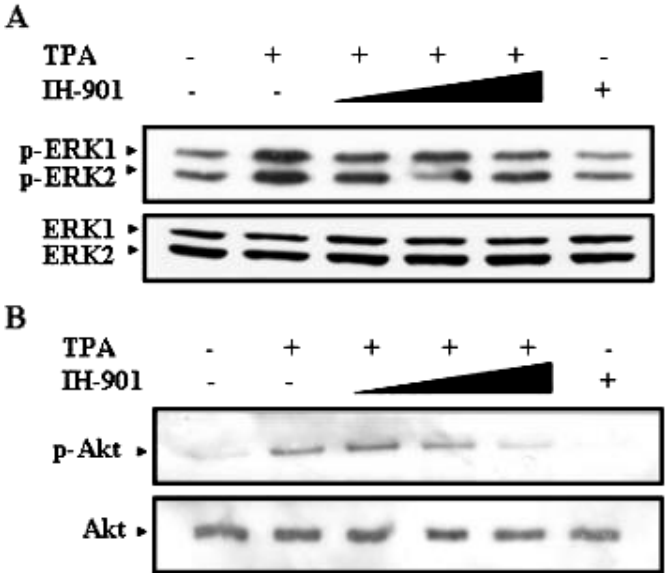

Fig. 4. Effects of IH-901 on TPA-induced phosphorylation of ERK and Akt in mouse skin. Female ICR mice were treated topically with acetone or with 1, 3 or 10 μmol of IH-901 30 min prior to TPA. Mice were killed 4 h after the TPA treatment; the phosphorylated form of ERK (**A**) or Akt (**B**) was detected by immunoblotting using phospho-specific antibodies. Data are representative of two independent experiments, which gave rise to a similar trend.

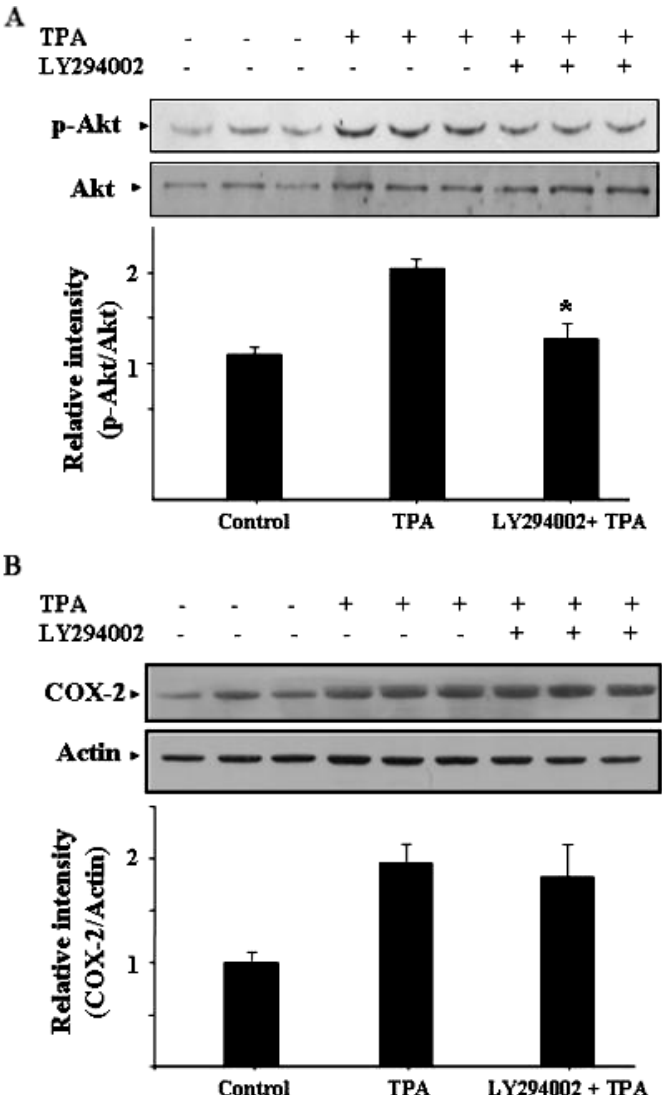

Fig. 5. Effects of LY294002 on Akt phosphorylation and COX-2 expression in mouse skin. Female ICR mice were treated topically with acetone or LY294002 (10 μmol) 30 min prior to 10 nmol TPA, and animals were killed 4 h after the TPA treatment. Protein was analyzed for phospho-Akt and COX-2 by immunoblotting. Quantification of phospho-Akt and COX-2 expressions was normalized to total Akt or actin using a densitometer. *Significantly different from the group treated with TPA alone ($P < 0.01$).

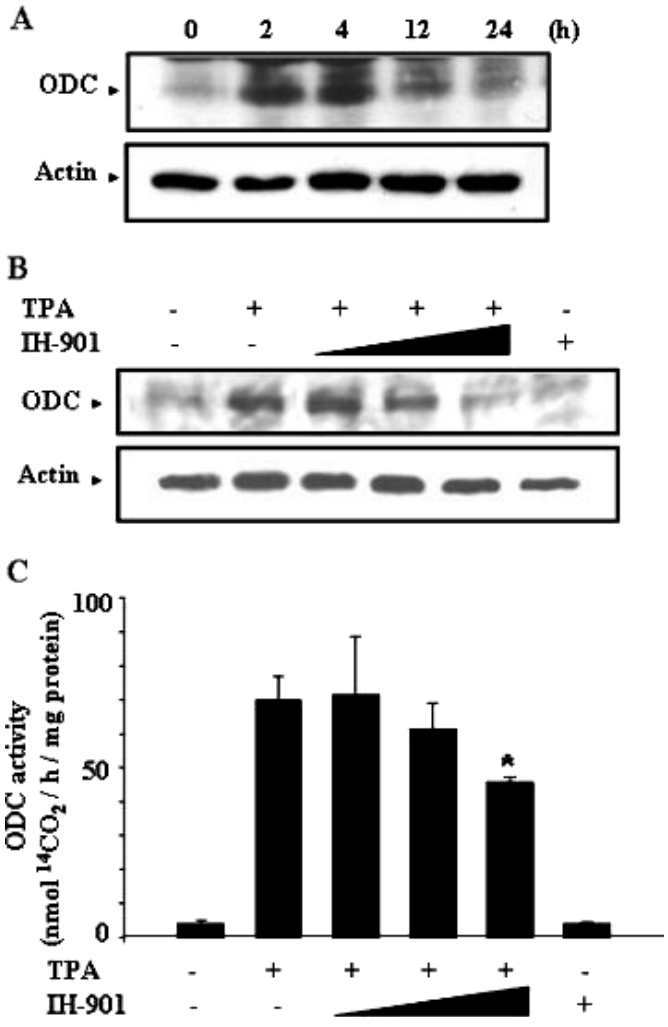

Fig. 6. Effects of IH-901 on TPA-induced ODC expression and activity in mouse skin. (**A**) Dorsal skins of female ICR mice were treated topically with acetone alone or with 10 nmol TPA in acetone for indicated time periods. Protein extracts (30 μg) were loaded onto a 12% SDS–polyacrylamide gel, electrophoresed, and subsequently transferred onto PVDF membrane. Immunoblots were probed with a monoclonal ODC antibody. (**B**) Mice were treated topically with acetone or with 1, 3 or 10 μmol of IH-901 30 min prior to 10 nmol TPA. Mice were killed 4 h after the TPA treatment for the measurement of ODC expression. (**C**) Female ICR mice were treated topically with 1, 3 or 10 μmol of IH-901 30 min prior to the application of 10 nmol TPA. The animals were killed 5 h after the TPA treatment, and epidermal ODC activity was measured. Data are expressed as the means ± SD obtained from 4 to 5 mice/group. *Significantly different from the TPA alone group ($P < 0.005$).

Effects of IH-901 on TPA-induced ODC activity and expression

ODC is a rate-limiting enzyme in the synthesis of polyamines that play pivotal roles in cell growth and proliferation (25). ODC is rapidly induced by many mitogenic stimuli including TPA and has been hence regarded as a biochemical hallmark of tumor promotion (26). As ODC expression peaked at ~4 h following TPA (10 nmol) treatment (Figure 6A), the effect of IH-901 on ODC was determined at this particular time point. When applied topically onto shaven backs of mice 30 min prior to TPA, IH-901 inhibited the epidermal ODC expression in a dose-dependent manner (Figure 6B). Likewise, the TPA-induced ODC activity was repressed by same doses of IH-901 (Figure 6C). IH-901 alone did not affect constitutive expression and activity of ODC.

Inhibitory effect of IH-901 on TPA-induced mouse skin tumor promotion

To examine whether IH-901 has an antitumor-promoting activity, the dorsal skin of mice was subjected to topical application of IH-901 prior to each TPA in the entire promotion stage. The first appearance of papillomas occurred at the eighth week of promotion. The DMBA-TPA group exhibited the 100% incidence of skin tumors with approximately 16 papillomas per mouse at 18th week. When IH-901 at a dose of 0.5 or 2.5 μmol was applied topically onto the shaven backs of mice 30 min prior to each TPA application, the average number of papillomas per mouse was reduced by 47 and 63%, respectively (Figure 7A). However, IH-901

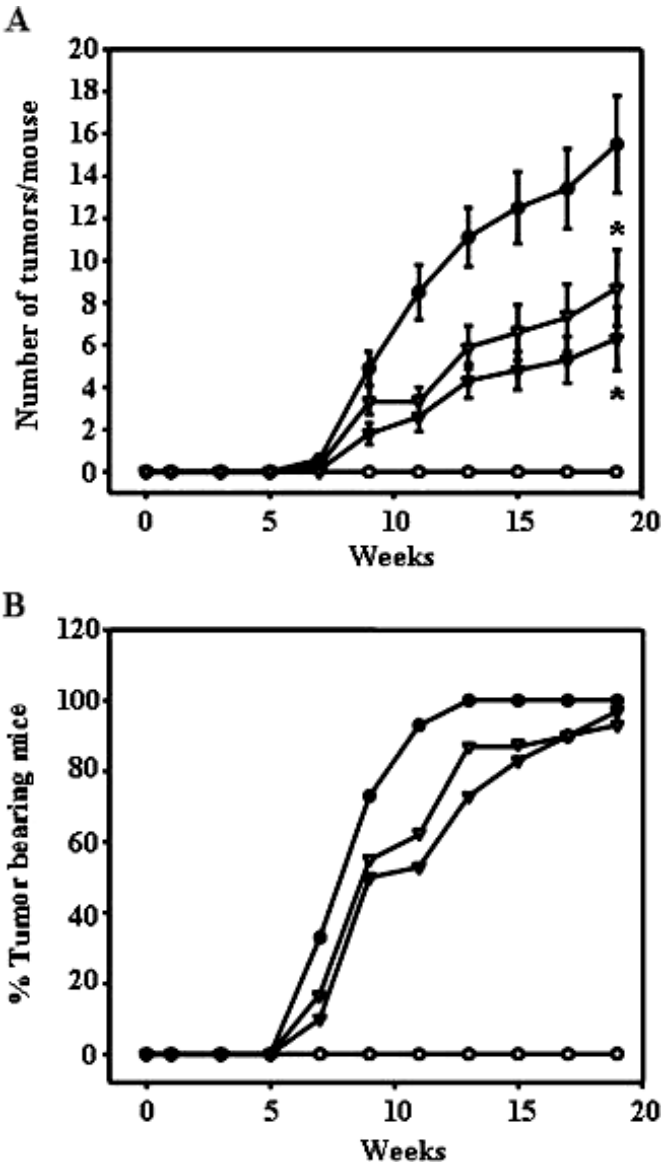

Fig. 7. Antitumor-promoting effects of IH-901 on mouse skin carcinogenesis. Female ICR mice were treated with 0.5 (△) or 2.5 (▼) μmol of IH-901 in 10% H_2O in acetone or the solvent alone (●) 30 min prior to each topical application of TPA (15 nmol) after initiation with DMBA (0.2 μmol). Control animals were treated with acetone in lieu of TPA (○). The asterisk indicates that the multiplicity of tumors is significantly lower than that observed in the DMBA-TPA group ($P < 0.01$).

pre-treatment did not significantly lower the percentage of tumor-bearing mice at the termination of the experiment (Figure 7B).

Discussion

In recent years considerable effort has been made to develop chemopreventive agents that could inhibit, retard or reverse the multistage carcinogenesis (27). Tumor promotion is closely linked to inflammation and oxidative stress (28,29), and it is hence likely that compounds with strong anti-inflammatory and anti-oxidative activities act as antitumor promoters as well.

Our previous studies have demonstrated the anti-inflammatory effects of several ginsenosides (Rb1, Rc, Re, Rg1 and Rg3) derived from *P.ginseng* (30). Topical application of each of these ginsenosides significantly attenuated ear edema induced by TPA. These ginsenosides also suppressed the expression of COX-2 and activation of NF-κB in the TPA-treated dorsal skin of mice (30). Of the ginsenosides tested, Rg3 was found to be most effective in terms of inhibiting TPA-induced ear edema, COX-2 expression and NF-κB activation. IH-901 is the major metabolite formed by intestinal bacteria from protopanaxadiol class ginseng saponins. It has been demonstrated that the *in vivo* anti-metastatic effects of some ginsenosides are mediated by this metabolite (11). IH-901 is known to be non-toxic, to inhibit glucose uptake by tumor cells (9), and to reverse multi-drug resistance in tumor cells (31). Moreover, IH-901 possesses chemopreventive potential, as evidenced by inhibition of genotoxicity and clastogenicity induced by benzo[*a*]pyrene (10). As an initial approach to evaluate the antitumor-promoting potential of IH-901, its effect on tumor promoter-induced inflammation was initially assessed by employing a mouse ear edema model. Topical application of IH-901 resulted in marked attenuation of TPA-induced inflammation in the mouse ear. In the subsequent experiment, the antitumor promotional activity of IH-901 was confirmed in a two-stage mouse skin carcinogenesis.

There is a growing body of compelling evidence that targeted inhibition of COX-2 expression or activity is valuable for not only alleviating inflammation, but also in the therapeutic prevention of cancer (32). Therefore, agents that interfere with the intracellular signaling mechanisms governing the transcription of COX-2 are considered to be potential chemopreventives. In our present study, topically applied IH-901 inhibited TPA-induced PGE_2 production almost completely at a dose that partially blocks COX-2 expression. This finding suggests that the antitumor-promoting effects of IH-901 can be attributed, in part, to inhibition of not only expression of COX-2 but also its catalytic activity. It is interesting to note that IH-901 barely affected the levels of the housekeeping enzyme COX-1. Since targeted inhibition of COX-2 without affecting the COX-1 expression is recognized as one of the most promising strategies for chemoprevention, IH-901 with selective COX-2 inhibitory activity has a therapeutic potential. It would be worthwhile comparing the anti-inflammatory as well as chemopreventive effects of IH-901 and its parental ginsenoside(s).

Control of *cox-2* induction involves a complex array of regulatory factors including NF-κB (33,34). Pre-treatment of the dorsal skin of mice with IH-901 inhibited the NF-κB DNA binding by blocking of p65 nuclear translocation and IκBα degradation. According to our previous study, topical application of the NF-κB inhibitor pyrrolidine dithiocarbamate resulted in the suppression of TPA-induced COX-2 expression in mouse skin (35). The data also support the notion that the naturally occurring anti-inflammatory agent IH-901 inhibits TPA-induced COX-2 expression in mouse skin, possibly by blocking NF-κB activation. Another transcription factor that plays an important role in controlling *cox-2* gene is activator protein-1 (AP-1). A role of AP-1 in COX-2 induction has been demonstrated in various cell lines (36–38). Topical application of IH-901 suppressed TPA-stimulated AP-1 activation in mouse skin (data not shown). Therefore, it is plausible that IH-901 inhibits COX-2 expression through not only inactivation of NF-κB but also other transcription factors including AP-1 in mouse skin.

The molecular signaling mechanisms involved in the induction of COX-2 as well as activation of transcription factors

including NF-κB in response to various external stimuli have not been fully clarified. One of the most extensively investigated intracellular signaling cascades involved in pro-inflammatory responses is the MAP kinase pathway. MAP kinases regulate NF-κB activation via multiple mechanisms. Our previous studies have demonstrated that the ultrapotent MEK1/2 inhibitor U0126 abolished the NF-κB binding activity and COX-2 expression in TPA-treated mouse skin (22), which supports the notion that ERK may play an important role in the signaling pathway mediating TPA-induced COX-2 expression and NF-κB activation in mouse skin. Therefore, we examined whether IH-901 could also block ERK activation by inhibiting phosphorylation of this MAP kinase. Topical application of IH-901 suppressed TPA-induced ERK phosphorylation in a dose-dependent manner. These results may provide the molecular basis for the inhibitory effect of IH-901 on NF-κB activation in TPA-treated mouse skin *in vivo*.

The serine/threonine kinase Akt is activated by numerous growth factors and many other stimuli (39). Activated Akt functions to promote cell survival by inhibiting apoptosis through its ability to phosphorylate and inactivate downstream targets (40). Recent reports have shown that Akt regulated COX-2 gene and protein expressions in phospho-Akt expressing endometrial cancer cells and inhibition of PI3-K with wortmannin and LY294002 blocked IκB phosphorylation, reduced NF-κB nuclear activity and COX-2 expression and induced apoptosis (41). In our current work, Akt was activated via phosphorylation in mouse skin in response to TPA treatment and inhibited by IH-901 pretreatment. However, LY294002 had no effect on COX-2 expression despite its ability to inhibit PI3-K activity. These findings suggest that the PI3K–Akt signaling pathway may not be a major target of IH-901 in its suppression of TPA-induced COX-2 expression in mouse skin. The utilization of genetically tractable mouse models, in which PI3K or Akt is functionally knocked out, would better test the validity of such conclusion.

ODC, a rate-limiting enzyme catalyzing biosynthesis of polyamines, is a biochemical hallmark of tumor promotion (42). Therefore, suppression of ODC induction during the carcinogenic process has been recognized as an important and commonly acceptable approach for the effective inhibition of tumor promotion (43). In the present study, TPA-induced ODC expression as well as enzyme activity was inhibited by

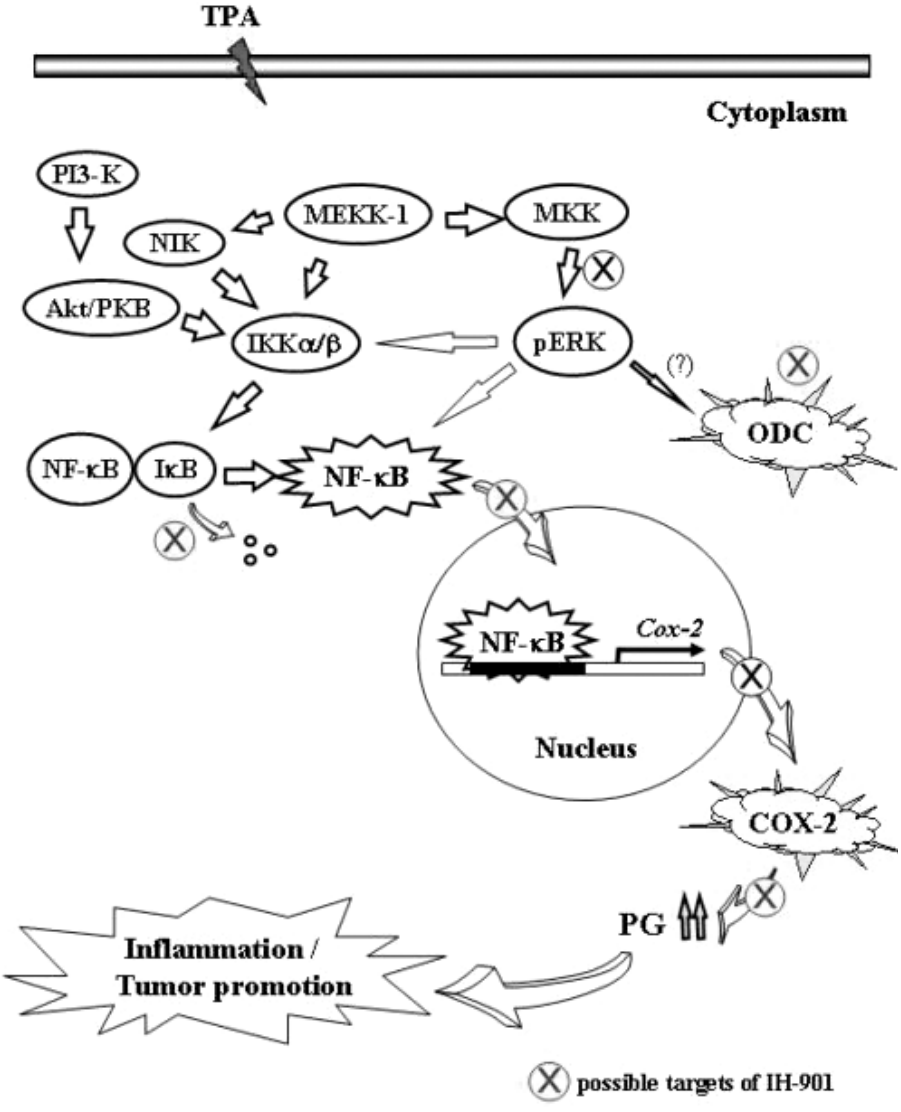

Fig. 8. Schematic representation of the possible targets of IH-901 and selected ginsenosides in the signal transduction pathway.

IH-901 pre-treatment in a dose-dependent manner. The molecular mechanisms underlying suppression of ODC expression and catalytic activity by IH-901 remains to be clarified. ODC induction is subjected to a complex array of cell-specific regulatory mechanisms that govern transcription, mRNA stability, translation and enzyme degradation (25). Kubota *et al*. (44) found that ODC over-expressing cells showed enhanced ERK activity, compared with the control transfectant. According to this study, the enhanced ERK activity in ODC over-expressing transfectant appears to be responsible for the neoplastic transformation and cell invasion observed in this genetically engineered cell line. Other investigators have reported that ODC over-expression stimulates MAP kinase expression and anchorage-independent growth in human breast cancer cells (45). MAP kinases have also been reported to be involved in the expression of ODC in leukemia cells (46) and in human ECV304 cells (47). Based on these findings, the persistent activation of the MAP kinase pathway, which is thought to have an important role in oncogenesis, is likely to cause elevated ODC expression. Pharmacologic inhibition of ERK by specific MEK1/2 blockers, such as U0126 and PD98059, prevented the induction of ODC (47), lending further support to the above supposition. Therefore, the induction of ODC would be down-stream of ERK activation. The association of another kinase Akt with ODC was also demonstrated in mouse leukemia (48). Additional studies will be necessary to clarify that the inhibitory effect of IH-901 on the TPA-induced ODC expression could be mediated through down-regulation of the ERK and/or Akt pathway.

The antitumor-promoting effect of IH-901 on mouse skin carcinogenesis was found to be more prominent in terms of reducing the multiplicity while the tumor incidence was inhibited to a lesser extent. This is not surprising if one considers the proapoptotic and anti-angiogenic properties of IH-901 (14–18). By eliminating the damaged cells in an early stage and by blocking the tumor-induced 'neovascularization', IH-901 is likely to prevent the tumor cells to spread out.

In conclusion, the present study demonstrates that IH-901 inhibits induction of COX-2 and ODC in TPA-treated mouse skin *in vivo*. Since improper and abnormal over-expression of COX-2 and ODC is implicated in the pathogenesis of various types of cancers, assessment of the effects of IH-901 on the expression of these two enzymes may be a useful surrogate biomarker for the evaluation of its activity in chemoprevention trials. Our findings that IH-901 inhibits TPA-induced COX-2 expression by blocking the ERK and NF-κB signaling cascades may provide a molecular basis for suppression of mouse skin tumor promotion as well as inflammation elicited by this chemopreventive phytochemical (Figure 8).

Acknowledgements

This study was supported by the National Research Laboratory (NRL) Grant awarded to Y.-J.S. from the Korea Institute of Science and Technology Evaluation and Planning (KISTEP), Ministry of Science and Technology, The Republic of Korea.

References

1. Attele,S.A., Wu,J.A. and Yuan,C.S. (1999) Ginseng pharmacology, multiple constitutes and multiple actions. *Biochem. Pharmacol*., **58**, 1685–1693.
2. Wang,Y., Wang,B.X., Liu,T.H., Minami,M., Nagata,T. and Ikejima,T. (2000) Metabolism of ginsenoside Rg_1 by intestinal bacteria. II. Immunological activity of ginsenoside Rg_1 and Rh_1. *Acta Pharmacol. Sin*., **21**, 792–796.
3. Akao,T., Kanaoka,M. and Kobashi,K. (1998) Appearance of compound K, a major metabolite of ginsenoside Rb_1 by intestinal bacteria, in rat plasma after oral administration—measurement of compound K by enzyme immunoassay. *Biol. Pharm. Bull*., **21**, 245–249.
4. Hasegawa,H., Sung,J.H., Matsumiya,S. and Uchiyama,M. (1996) Main ginseng saponin metabolites formed by intestinal bacteria. *Planta Med*., **62**, 453–457.
5. Karikura,M., Miyase,T., Tanizawa,H., Taniyama,T. and Takino,Y. (1991) Studies on absorption, distribution, excretion and metabolism of ginseng saponins. VII. Comparison of the decomposition modes of ginsenoside-Rb_1 and -Rb_2 in the digestive tract of rats. *Chem. Pharm. Bull*., **39**, 2357–2361.
6. Sung,J.H., Hasegawa,H., Matsumiya,S., Uchiyama,M., Ha,J.Y., Lee,M.S. and Huh,J.D. (1995) Metabolism of ginseng saponins by human intestinal bacteria. *Korean J. Pharmacogen*., **26**, 360–367.
7. Akao,T., Kida,H., Kanaoka,M., Hattori,M. and Kobashi,K. (1998) Intestinal bacterial hydrolysis is required for the appearance of compound K in rat plasma after oral adiministration of ginsenoside Rb_1 from *Panax ginseng*. *J. Pharm. Pharmacol*., **50**, 1155–1160.
8. Hasegawa,H., Sung,J.H. and Benno,Y. (1997) Role of human intestinal *Prevotella oris* in hydrolyzing ginseng saponins. *Planta Med*., **63**, 436–440.
9. Hasegawa,H., Matsumiya,S., Uchiyama,M., Kurokawa,T., Inouye,Y., Kasai,R., Ishibashi,S. and Yamasaki,K. (1994) Interactions of ginseng extract, ginseng separated fractions, and some triterpenoid saponins with glucose transporters in sheep erythrocytes. *Planta Med*., **60**, 240–243.
10. Lee,B.H., Lee,S.J., Hui,J.H., Lee,S., Sung,J.H., Huh,J.D. and Moon,C.K. (1998) *In vitro* antigenotoxic activity of novel ginseng saponin metabolites formed by intestinal bacteria. *Planta Med*., **64**, 500–503.
11. Wakabayashi,C., Hasegawa,H., Murata,J. and Saiki,I. (1997) *In vivo* antimetastatic action of ginseng protopanaxadiol saponins is based on their intestinal bacterial metabolites after oral administration. *Oncol. Res*., **9**, 411–417.
12. Hasegawa,H., Sung,J.H. and Huh,J.D. (1997) Ginseng intestinal bacterial metabolite IH901 as a new anti-metastatic agent. *Arch. Pharm. Res*., **20**, 539–544.
13. Hasegawa,H. and Uchiyama,M. (1998) Antimetastatic efficacy of orally administered ginsenoside Rb_1 is dependent on intestinal bacterial hydrolyzing potential and significance of treatment with an active bacterial metabolite. *Planta Med*., **84**, 696–700.
14. Oh,S.H., Yin,H.Q. and Lee,B.H. (2004) Role of the Fas/Fas ligand death receptor pathway in ginseng saponin metabolite-induced apoptosis in HepG2 cells. *Arch. Pharm. Res*., **27**, 402–406.
15. Oh,S.H. and Lee,B.H. (2004) A ginseng saponin metabolite-induced apoptosis in HepG2 cells involves a mitochondria-mediated pathway and its downstream caspase-8 activation and Bid cleavage. *Toxicol. Appl. Pharmacol*., **194**, 221–229.
16. Choi,H.H., Jong,H.S., Park,J.H., Choi,S., Lee, J.W., Kim,Y.Y., Otsuki,T., Namba,M. and Bang,Y.J. (2003) A novel ginseng saponin metabolite induces apoptosis and down-regulates fibroblast growth factor receptor 3 in myeloma cells. *Int. J. Oncol*., **23**, 1087–1093.
17. Lee,S.J., Ko,W.G., Kim,J.H., Sung,J.H., Moon,C.K. and Lee,B.H. (2000) Induction of apoptosis by a novel intestinal metabolite of ginseng saponin via cytochrome *c*-mediated activation of caspase-3 protease. *Biochem. Pharmacol*., **60**, 677–685.
18. Wakabayashi,C., Murakami,K., Hasegawa,H., Murata,J. and Saiki,I. (1998) An intestinal bacterial metabolite of ginseng protopanaxadiol saponins has the ability to induce apoptosis in tumor cells. *Biochem. Biophys. Res. Commun*., **246**, 725–730.
19. Fujiki,H., Suganuma,M., Komori,A., Yatsunami,J., Okabe,S., Ohta,T. and Sueoka,E. (1994) A new tumor promotion pathway and its inhibitors. *Cancer Detect. Prev*., **18**, 1–7.
20. Han,S.S., Keum,Y.-S., Seo,H.J., Chun,K.-S., Lee,S.S. and Surh,Y.-J. (2001) Capsaicin suppresses phorbol ester-induced activation of NF-κB/Rel and AP-1 transcription factors in mouse epidermis. *Cancer Lett*., **164**, 119–126.
21. Chun,K.-S., Park,K.-K., Lee,J., Kang,M. and Surh,Y.-J. (2002) Inhibition of mouse skin tumor promotion by anti-inflammatory diarylheptanoids derived from *Alpinia oxyphylla* Miquel (Zingiberaceae). *Oncol. Res*., **13**, 37–45.
22. Chun,K.S., Keum,Y.S., Han,S.S., Song,Y.S., Kim,S.H. and Surh,Y.J. (2003) Curcumin inhibits phorbol ester-induced expression of cyclooxygenase-2 in mouse skin through suppression of extracellular

signal-regulated kinase activity and NF-κB activation. *Carcinogenesis*, **24**, 1515–1524.
23. Romashkova,J.A. and Makarov,S.S. (1999) NF-κB is a target of AKT in anti-apoptotic PDGF signalling. *Nature*, **401**, 86–90.
24. Xie,P., Browning,D.D., Hay,N., Mackman,N. and Ye,R.D. (2000) Activation of NF-kappa B by bradykinin through a Galpha(q)- and Gbeta gamma-dependent pathway that involves phosphoinositide 3-kinase and Akt. *J. Biol. Chem.*, **275**, 24907–24914.
25. Heby,O. and Persson,L. (1990) Molecular genetics of polyamine synthesis in eukaryotic cells. *Trends Biochem. Sci.*, **15**, 153–158.
26. O'Brien,T.G., Simsiman,R.C. and Boutwell,R.K. (1975) Induction of the polyamine-biosynthetic enzymes in mouse epidermis by tumor-promoting agents. *Cancer Res.*, **35**, 1662–1670.
27. Weinstein,I.B. (1991) Cancer prevention: recent progress and future opportunities. *Cancer Res.*, **51** (18 suppl.), 5080s–5085s.
28. DiGiovanni,J. (1992) Multistage carcinogenesis in mouse skin. *Pharmacol. Ther.*, **54**, 63–128.
29. Bhimani,R.S., Troll,W., Grunberger,D. and Frenkel,K. (1993) Inhibition of oxidative stress in HeLa cells by chemopreventive agents. *Cancer Res.*, **53**, 4528–4533.
30. Surh,Y.-J., Lee,J.Y., Choi,K.J. and Ko,S.R. (2002) Effects of selected ginsenosides on phorbol ester-induced expression of cyclooxygenase-2 and activation of NF-κB and ERK1/2 in mouse skin. *Ann. N. Y. Acad. Sci.*, **973**, 396–401.
31. Hasegawa,H., Sung,J.H., Matsumiya,S., Uchiyama,M., Inouye,Y., Kasai,R. and Yamasaki,K. (1995) Reversal of daunomycin and vinblastine resistance in multidrug-resistant P388 leukemia *in vitro* through enhanced cytotoxicity by triterpenoids. *Planta Med.*, **61**, 409–413.
32. Subbaramaiah,K. and Dannenberg,A.J. (2003) Cyclooxygenase 2: a molecular target for cancer prevention and treatment. *Trends Pharmacol. Sci.*, **24**, 96–102.
33. D'Acquisto,F., Iuvone,T., Rombola,L., Sautebin,L., Di Rosa,M. and Carnuccio,R. (1997) Involvement of NF-κB in the regulation of cyclooxygenase-2 protein expression in LPS-stimulated J774 macrophages. *FEBS Lett.*, **418**, 175–178.
34. Kojima,M., Morisaki,T., Izuhara,K., Uchiyama,A., Matsunari,Y., Katano,M. and Tanaka,M. (2000) Lipopolysaccharide increases cyclooxygenase-2 expression in a colon carcinoma cell line through nuclear factor-κB activation. *Oncogene*, **19**, 1225–1231.
35. Surh,Y.-J., Chun,K.-S., Cha,H.-H., Han,S.S., Keum,Y.-S., Park, K.-K. and Lee,S.S. (2001) Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-κB activation. *Mutat. Res.*, **480/481**, 243–268.
36. Guo,Y.S., Hellmich,M.R., Wen,X.D. and Townsend,C.M. Jr (2001) Activator protein-1 transcription factor mediates bombesin-stimulated cyclooxygenase-2 expression in intestinal epithelial cells. *J. Biol. Chem.*, **276**, 22941–22947.
37. Subbaramaiah,K., Norton,L., Gerald,W. and Dannenberg,A.J. (2002) Cyclooxygenase-2 is overexpressed in HER-2/neu-positive breast cancer: evidence for involvement of AP-1 and PEA3. *J. Biol. Chem.*, **277**, 18649–18857.
38. Subbaramaiah,K., Lin,D.T., Hart,J.C. and Dannenberg,A.J. (2001) Peroxisome proliferator-activated receptor gamma ligands suppress the transcriptional activation of cyclooxygenase-2. Evidence for involvement of activator protein-1 and CREB-binding protein/p300. *J. Biol. Chem.*, **276**, 2440–2448.
39. Neri,L.M., Borgatti,P., Capitani,S. and Martelli,A.M. (2002) The nuclear phosphoinositide 3-kinase/AKT pathway: a new second messenger system. *Biochim. Biophys. Acta*, **1584**, 73–80.
40. Chang,F., Lee,J.T., Navolanic,P.M., Steelman,L.S., Shelton,J.G., Blalock,W.L., Franklin,R.A. and McCubrey,J.A. (2003) Involvement of PI3K/Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy. *Leukemia*, **17**, 590–603.
41. St-Germain,M.E., Gagnon,V., Parent,S. and Asselin,E. (2004) Regulation of COX-2 protein expression by Akt in endometrial cancer cells is mediated through NF-κB/IκB pathway. *Mol. Cancer*, **3**, 7.
42. Auvinen,M., Paasinen,A., Andersson,L.C. and Holtta,E. (1992) Ornithine decarboxylase activity is critical for cell transformation. *Nature*, **360**, 355–358.
43. Pegg,A.E., Shantz,L.M. and Coleman,C.R. (1995) Ornithine decarboxylase as a target for chemoprevention. *J. Cell Biol.*, **22**, 132–138.
44. Kubota,S., Kiyosawa,H., Nomura,Y., Yamada,T. and Seyama,Y. (1997) Ornithine decarboxylase overexpression in mouse 10T1/2 fibroblasts: cellular transformation and invasion. *J. Natl Cancer Inst.*, **89**, 567–571.
45. Manni,A., Wechter,R., Gilmour,S., Verderame,M.F., Mauger,D. and Demers,L.M. (1997) Ornithine decarboxylase over-expression stimulates mitogen-activated protein kinase and anchorage-independent growth of human breast epithelial cells. *Int. J. Cancer*, **70**, 175–182.
46. Flamigni,F., Facchini,A., Capanni,C., Stefanelli,C., Tantini,B. and Caldarera,C.M. (1999) p44/42 mitogen-activated protein kinase is involved in the expression of ornithine decarboxylase in leukaemia L1210 cells. *Biochem. J.*, **341**, 363–369.
47. Flamigni,F., Facchini,A., Giordano,E., Tantini,B. and Stefanelli C. (2001) Signaling pathways leading to the induction of ornithine decarboxylase: opposite effects of p44/42 mitogen-activated protein kinase (MAPK) and p38 MAPK inhibitors. *Biochem. Pharmacol.*, **61**, 25–32.
48. Flamigni,F., Marmiroli,S., Capanni,C., Stefanelli,C., Guarnieri,C. and Caldarera,C.M. (1997) Phosphatidylinositol 3-kinase is required for the induction of ornithine decarboxylase in leukemia cells stimulated to growth. *Biochem. Biophys. Res. Commun.*, **239**, 729–733.

Received June 6, 2004; revised October 2, 2004; accepted October 10, 2004