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Anti-tumor Promoting Potential of *Artemisia asiatica*: Suppression of Proinflammatory Enzyme Induction in Phorbol Ester-treated Mouse Skin

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Artemisia asiatica Nakai (Asteraceae) has been used in traditional oriental medicine for the treatment of inflammation and other disorders. As an initial approach towards determining the possible anti-tumor promoting potential of *A. asiatica,* the effects of the ethanol extract of this plant on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) were examined in female ICR mice. Pretreatment of the shaven back of mice with *A. asiatica* 30 min prior to topical application of TPA inhibited expression of these enzymes in a dose-dependent manner. Moreover, *A. asiatica* treatment attenuated TPA-stimulated epidermal NF-κB activation, which was associated with its blockade of degradation of the inhibitory protein IκBα and also of subsequent translocation of the p65 subunit to nucleus. Our findings that *A. asiatica* inhibits TPA-induced COX-2 and iNOS expression by blocking the NF-κB signaling cascades may provide molecular basis for suppression of mouse skin inflammation and tumor promotion by this chemopreventive plant.

Key Words: Anti-tumor promotion, Cyclooxygenas-2, Inducible nitric oxide synthase, Mouse skin, NF-κB

INTRODUCTION

Carcinogenesis is a multi-stage process in which the summation of genetic and/or epigenetic changes produces phenotypically distinct characteristics of malignant cells. The multi-stage carcinogenesis can be divided into 3 broad stages, i.e., initiation, promotion and progression. Initiation is an irreversible event in nature which generally results from DNA damage produced by a metabolically activated genotoxic carcinogen. Tumor promotion, by contrast, is defined as a reversible, epigenetic and relatively lengthy process which represents clonal expansion of the initiated phenotype to form a population of preneoplastic cells.[1,2] Progression occurs in a relatively predictable sequence[2] and produces a new clone of malignant cells with increased proliferative capacity, invasiveness, and metastatic protential.

Tumor promotion accompanies a generation of free radicals and reactive oxygen species (ROS), depletion

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of the cellular antioxidant capacity, acute inflammation characterized by skin edema and hyperplasia, and induction of proinflammatory enzymes such as cyclooxygenase (COX) and inducible nitric oxide synthase (iNOS).[3] In many instances, the chemopreventive activities of phytochemicals correlate with their anti-inflammtory and/or antioxidative properties.[4]

The iNOS and COX-2 pathways share a number of similarities. They regulate several important physiological effects (e.g., antiplatelet activity, vasodilation, cytoprotection, etc.). On the other hand, in inflammatory setting, iNOS and COX-2 are induced in a variety of cells, resulting in the production of large amounts of proinflammatory and cytotoxic NO and prostaglandins respectively.

Nuclear factor-κB (NF-κB) is a ubiquitous, pleiotropic, multi-subunit eukaryotic transcription factor existing as either homo- or heterodimer of various subunits of Rel family proteins referred to as p50, p52, p65 (RelA), c-Rel, and Rel-B.[5~7] These complexes are activated by antigens, viruses, bacteria, and inflammatory cytokines, and result in transcriptional initiation of a diverse set of genes, including iNOS and COX-2, whose promoters have NF-κB-binding consensus sequences. The importance of NF-κB in mediating inflammatory events is also evident from experiments utilizing knock-out animals, suggesting that this transcription factor is a relevant target for potential anti-inflammatory agents.[8] NF-κB is normally sequestered in the cytoplasm by binding to the inhibitory protein, IκB. When the cells are exposed to external stimuli such as mitogens, inflammatory cytokines, ultraviolet irradiation, ionizing radiation, viral proteins, bacterial lipopolysaccharides (LPS) and reactive oxygen species (ROS), NF-κB is activated through phosphorylation of IkB protein at specific serine residues by a protein kinase complex called IkB kinase (IKK). Upon phosphorylation, IκBα undergoes ubiquitination and rapid degradation by 26S proteasomes.[9] The activated NF-κB then translocates to the nucleus where it binds to a specific consensus motif present in the promoter or the enhancer region of target genes.[10] Constitutively active NF-κB has been detected in Hodgkin's lymphoma,[11] human colonic adenoma,[12] and head and neck carcinoma.[13] Moreover, κB-binding activity increased in skin papillomas and squamous cell carcinomas produced by a two-stage mouse skin carcinogenesis protocol.[14] Therefore, attenuation of NF-κB activation is another plausible strategy to prevent cancer.

Artemisia asiatica (Asteraceae) has been frequently used in traditional oriental medicine for the treatment of such diseases as inflammation, cancer, and microbial infections. Its pharmacologically active ingredient eupatilin (5,7-dihydroxy-3,4,6-tri-methoxy-flavone) has been reported to selectively inhibit 5-lipoxygenase and to possess potent anti-gastric activities.[15] Recent studies from this laboratory have revealed that eupatilin induces apoptosis in cultured human promyelocytic leukemia HL-60 cells.[16] Since tumor promotion is closely related to inflammatory processes which can stimulate the proliferation of initiated cells, *A. asiatica* with substantial anti-inflammatory activity is anticipated to have anti-tumor promotional potential.

As one step towards evaluating the anti-tumor promotional activity of *A. asiatica,* we have determined its effects on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced expression of COX-2 and iNOS. *A. asiatica* was also tested for its possible inhibition of TPA-induced activation of NF-κB which is known to regulate expression both COX-2 and iNOS.[17]

MATERIALS AND METHODS

1) Chemicals

The ethanol extract of *A. asiatica* was obtained from Dong-A Pharm. Co. Ltd., Korea. TPA was purchased from Alexis Biochemicals (San Diego, CA, USA). All other chemicals were obtained in the purest forms available commercially.

2) Animals

Six-week-old female ICR mice were supplied from the Dae-Han Korea Experimental Animal Center

(Daejon, Korea). The animals were housed in climate-controlled quarters at 24±1°C at 50% humidity with a 12-h light/12-h dark cycle. The dorsal side of skin was shaved using an electric clipper, and only those animals in the resting phase of the hair cycle were used in all experiments.

3) Western blot analysis

The female ICR mice were topically treated on their shaven backs with indicated amounts of the ethanol extract of *A. asiatica* dissolved in 200μl acetone 30 min before 10 nmole TPA treatment and were killed by cervical dislocation at the indicated time points. For isolation of protein from mouse skin, the skin was excised, and the fat was removed on ice, immediately placed in liquid nitrogen and pulverized in mortar. The pulverized skin was homogenized on ice for 20 sec 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 DTT, 1 mM Na_3VO_4, protease inhibitor cocktail tablet (Roche Molecular Biochemicals, Mannheim, Germany)] for 10 min. The lysate was centrifuged at 12,000 *g* for 20 min, and supernatant was collected. The amount of protein contained in the supernatant was determined by using the Bicinchoninic Acid (BCA) protein assay kit (Pierce, Rockford, IL, USA). Supernatant containing 30μg protein was boiled in SDS sample loading buffer for 5 min before electrophoresis on 12% SDS-polyacrylamide gel. After electrophoresis for 2 h, proteins in the gel were transferred to PVDF membrane (Gelman Laboratory, Ann Arber, MI, USA), 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 corresponding primary antibodies. COX-2 polyclonal antibody purchased from Cayman chemical (Ann Arbor, MI, USA) was used at dilution of 1 : 1000. Polyclonal antibodies against iNOS and IκBα were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and used at 1 : 1000 dilution. p65 polyclonal antibody from Zymed Laboratories Inc., (San Francisco, CA, USA) was used at dilution of 1 : 1,000. The blots were rinsed three times with PBST buffer for 5 min each. Washed blots were incubated with 1 : 5,000 dilution of horseradish peroxidase conjugated-secondary antibody (Zymed Laboratories Inc., San Francisco, CA, USA) and then washed again three times with PBST buffer for 5 min each. The transferred proteins were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech Inc., Buckinghamshire, UK).

4) Preparation of nuclear extracts

The nuclear extract from mouse skin was prepared as described previously.[18] Briefly, 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 $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.1 mM phenylmethylsulfonylfluoride (PMSF)]. To the homogenate was added 125μl of 10% Nonidet P-40 (NP-40) solution after 15 min incubation on ice, and the mixture was then centrifuged for 30 sec at 12,000 rpm. The supernatant was collected, and the pelleted nuclei were washed once with 400μl of buffer A plus 25μl of 10% NP-40 and centrifuged again. The washed nuclei were resuspended in 150μl 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], incubated for 20 min on ice, and centrifuged for 5 min at 12,000 rpm. The supernatant containing nuclear proteins was collected and stored at -70°C after determination of protein concentrations.

5) Electrophoretic mobility shift assay (EMSA)

EMSA was performed using a DNA-protein binding detection kit (GIBCO BRL, Grand Island, NY, USA) according to the manufacturer's protocol. In brief, 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.,

Buckinghamshire, UK). The binding reaction was carried out in 25μl of the mixture containing 5μl of incubation buffer [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT 1 mM EDTA, 4% (v/v) glycerol, and 0.1 mg/ml sonicated salmon sperm DNA], 10μg of nuclear extracts, and 100,000 cpm of the labeled probe. After 50 min incubation at room temperature, 2μl of 0.1% bromophenol blue was added, and samples were electrophoresed through a 6% nondenaturing polyacrylamide gel at 150 V in a cold room for 2 h. Finally, the gel was dried under vacuum at 80°C and exposed to an X-ray film at -70°C.

RESULTS

Since tumor promotion is closely related to inflammation, the antitumor promoting effect of *A. asiatica* which has been used to treat inflammatory diseases is expected. As an initial attempt to verify the anti-inflmmatory activity of *A. asiatica*, its effect on TPA-induced COX-2 expression was examined. Topical application of TPA to mouse skin led to an transient increase in epidermal COX-2 expression (data not shown). The dose-dependent increase in COX-2 expression was observed with TPA at doses ranging from 2.5 to 25 nmole (Fig. 1). Since COX-2 expression peaked at about 4 h after TPA-stimulation (data not shown), the effect of *A. asiatica* on COX-2 was determined at this time point. Immunoblotting of COX-2 protein indicated that *A. asiatica* reduced the expression of this enzyme in a dose-dependent manner (Fig. 2). Besides suppressing COX-2 expression, *A. asiatica* markedly inhibited TPA-stimulated iNOS protein expression (Fig. 2).

Because activation of NF-κB is recognized to be critical for resulting induction of both COX-2 and iNOS by TPA or other inflammatory cytokines, we determined whether *A. asiatica* could suppress NF-κ

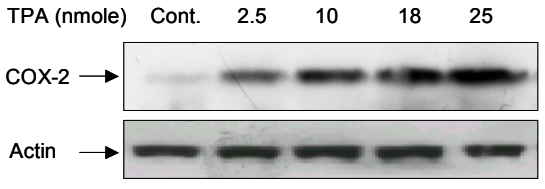

Fig. 1. TPA induced COX-2 protein expression in mouse skin. Dorsal skins of female ICR mice were treated topically with acetone alone or with various dose of TPA in acetone for 4 h. Protein extracts (30μg) were loaded onto a 12% SDS-polyacrylamide gel, electrophoresed, and subsequently transferred onto PVDF membrane. Immunoblots were a probed with COX-2 antibody.

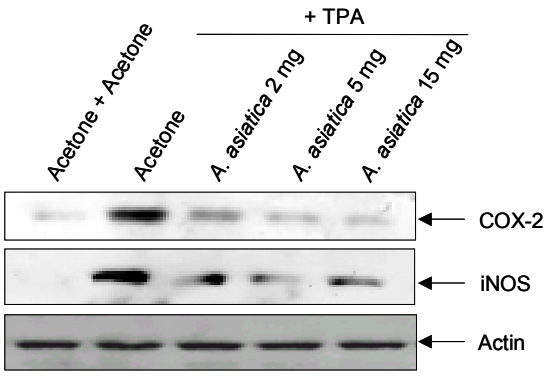

Fig. 2. Inhibitory effect of *A. asiatica* on phorbol ester-induced COX-2 expression. Female ICR mice were treated topically with 0.2 ml acetone or *A. asiatica* in the same vol of acetone 30 min prior to 10 nmole TPA, and animals were killed 4 h. Total protein was analyzed for COX-2 by immunoblotting.

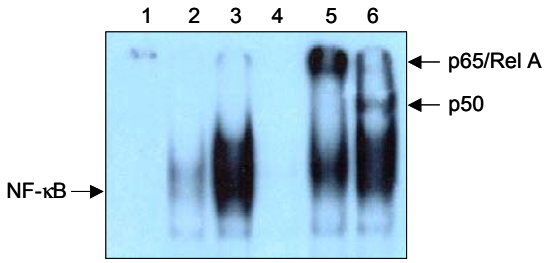

Fig. 3. Immunoreactivity of mouse epidermal NF-κB. Nuclear extracts from TPA-treated mice were incubated with antibodies against the NF-κB subunits p50 or p65 as described under Material and Methods. Lane 1, probe only; lane 2, acetone control; lane 3, TPA (10 nmole) alone; lane 4, lane 3+excess cold probe; lane 5, lane 3+p65 antibody; lane 6, lane 3+p50 antibody

B activation in TPA-treated mouse skin. The kinetic

studies demonstrated that TPA-induced NF-κB activation was detectable as early as 30 min after the treatment and peaked at 1 h.[18)]

The specificity of protein-DNA complexes was verified by competition with the excess unlabeled oligonucleotide (Fig. 3). We then analyzed the composition of the NF-κB complex. Antibodies against NF-κB subunits p65 and p50 were added to the nuclear extract of epidermis stimulated with TPA. The migration of NF-κB-DNA complex in EMSA was retarded completely by addition of anti-p65 (Fig. 3). Similar retardation was observed with anti-p50 (Fig. 3). These findings indicate that TPA-induced NF-κB complexes consisted at least of p65 and p50. Pretreatment of mouse skin with *A. asiatica* dose-dependently inhibited the DNA binding activity of epidermal NF-kB (Fig. 4A). *A. asiatica* alone did not influence constitutive NF-κB activation (Fig. 4B).

A key step of NF-κB activation is the dissociation of IkB, which is mediated through phosphorylation and subsequent proteolytic degradation of this inhibitory subunit. To determine whether the inhibitory action of *A. asiatica* towards NF-κB was due to its effect on IκBα degradation, the cytoplasmic level of IκBα was determined by Western blot analysis. Treatment with TPA led to the rapid degradation of IκBα, which was strongly inhibited by *A. asiatica* (Fig. 5). We also measured the level of p65, which is the functionally active subunit of NF-κB, in both cytoplasm and nucleus. Upon TPA treatment, the level of p65 declined in the cytoplasm with a concurrent increase in the nucleus which was repressed by *A. asiatica* pretreatment (Fig. 5). These results indicate that *A. asiatica* inhibits the TPA-induced translocation of p65 to the nucleus through blockade of TPA-dependent degradation of IκBα.

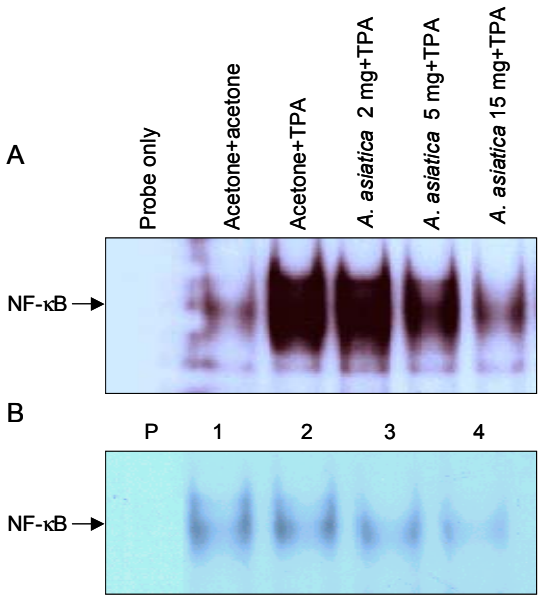

Fig. 4. A) Effect of *A. asiatica* on NF-κB activation in mouse skin treated with TPA. Dorsal skins of mice were treated topically with the indicated doses of *A. asiatica* 30 min prior to topical application of 10 nmole TPA. Mice were killed 1 h after the TPA treatment (acetone for the control), and epidermal nuclear extracts were prepared and incubated with radiolabelled oligonucleotides containing the NF-κB consensus sequence for analysis by the EMSA. B) Effect of *A. asiatica* on constitutive NF-κB activation in mouse skin. Mice were treated for 30 min with the indicated doses of *A. asiatica,* and epidermal unclear extracts and analyzed by EMSA. Lane 1, acetone alone; lane 2, *A. asiatica* 2 mg alone; lane 3, *A. asiatica* 5 mg alone; lane 4, *A. asiatica* 15 mg alone, P, probe only.

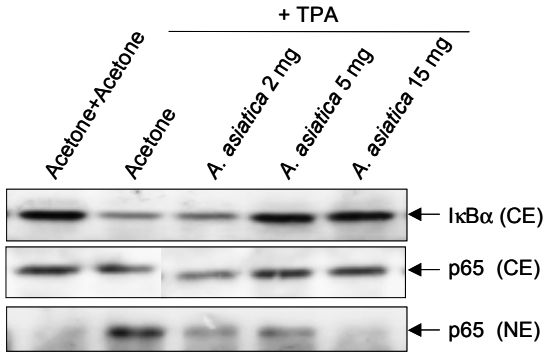

Fig. 5. Effect of *A. asiatica* on degradation of IκBα and nuclear translocation of p65. Nuclear and cytoplasmic extracts from TPA (10 nmole)-stimulated mouse skin treated 10 nmole TPA for 1 h, with and without *A. asiatica* (2, 5, or 15 mg) pretreatment, were assayed for p65 and for IκBα, respectively by Western blot analysis. NE, nuclear extract; CE, cytoplasmic extract.

DISCUSSION

One of the most promising approaches to reduce the risk of cancer is chemoprevention.[19,20] An attractive strategy of chemoprevention is the use of naturally occurring substances to prevent the occurrence and subsequent development of cancer by inhibiting, retarding, or reversing the process of multi-stage carcinogenesis.[21] Chemopreventers are divided into two major subgroups, i.e., blocking agents that can inhibit the initiation step and suppressing agents that can interfere with either the promotion or the progression step.[22] Among three carcinogenesis processes, the promotion stage seems to be the most appropriate and practical target for chemoprevention. Since tumor promotion is a reversible and requires repeated and prolonged exposure to promoting agents such as TPA, it would be more important to identify chemopreventive agents that can suppress the promotion, rather than those target the initiation and the progression stages.

Plants of the *Astraceae* family have been widely used in dietary cuisines and also in traditional oriental medications without any serious adverse reactions. The standardized formulation prepared from the ethanol extract of *A. asiatica* possesses antioxidative and anti-inflammatory activities, which contribute to its protective effects against experimentally induced gastric damage[23] and pancreatities[24] and also anti-tumor promoting activities.[25] Its pharmacologically active ingredient is the flavonoid eupatilin, which has been reported to induce apoptotic death of cultured human promyelocytic leukemia (HL-60) cells.[16]

The present study demonstrates that *A. asiatica* exhibits inhibitory effects on TPA-induced expression of COX-2 or iNOS in mouse skin. These findings provide a significant molecular basis as to how *A. asiatica* exerts anti-tumor promoting as well as anti-inflammatory activity.

Recently, it was found that overexpression of either COX-2 or iNOS may be intimately involved in the pathogenesis of many disease, including colon cancer.[26] Although numerous agents have been synthesized as effective inhibitors of COX-2 or iNOS, an alternative approach might be taken to develop combined agents that suppress transcription of these two enzymes more effectively than does either alone.[27~30]

Regulation of COX-2 and iNOS induction has been considered to involve a complex array of regulatory factors including NF-κB.[31,32] Pre-treatment with *A. asiatica* inhibited NF-κB activation induced by TPA in a dose dependent manner. In order to elucidate molecular mechanisms underlying suppression of TPA-induced NF-κB activation by *A. asiatica*, we compared the levels of p65, the functionally active NF-κB subunit as well as those of IκBα in cytoplasmic and/or nuclear extracts from mouse skin. Topical application of 10 nmole TPA resulted in almost complete disappearance of IκBα in the cytoplasm, which was accompanied by elevated levels of nuclear p65. *A. asiatica* pretreatment significantly suppressed TPA-induced degradation of IκBα, thereby blocking nuclear translocation of p65.

In summary, this study demonstrates that the ethanol extract of *A. asiatica* inhibits TPA-induced expression of inflammatory enzymes such as COX-2 and iNOS, possibly via suppression of NF-κB activation. Taken together, our data presented herein suggest that *A. asiatica* has the chemopreventive potential. Additional studies will be required to clarify the upstream intracellular signal-transduction pathways that can be influenced by the ethanolic extract of *A. asiatica* and to elucidate the structural identity of the active principles responsible for the chemopreventive effects that this medicinal plant exerts.

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