Humulone inhibits phorbol ester-induced COX-2 expression in mouse skin by blocking activation of NF-κB and AP-1: IkB kinase and c-Jun-N-terminal kinase as respective potential upstream targets

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Humulone, a bitter acid derived from hop (Humulus lupulus L.), possesses antioxidant, anti-inflammatory and other biologically active activities. Although humulone has been reported to inhibit chemically induced mouse skin tumor promotion, the underlying mechanisms are yet to be elucidated. Since an inappropriate over-expression of cyclooxygenase-2 (COX-2) is implicated in carcinogenesis, we investigated effects of humulone on COX-2 expression in mouse skin stimulated with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA). Topical application of humulone (10 μmol) significantly inhibited TPA-induced epidermal COX-2 expression. Humulone also diminished TPA-induced DNA binding of nuclear factor-κB (NF-κB) and activator protein-1 (AP-1). Pre-treatment with humulone attenuated TPA-induced phosphorylation of p65 and nuclear translocation of NF-κB subunit proteins. Humulone blunted TPA-induced activation of inhibitory kappaB (IκB) kinase (IKK) in mouse skin, which accounts for its suppression of phosphorylation and subsequent degradation of IκBα. An in vitro kinase assay revealed that humulone could directly inhibit the catalytic activity of IKKβ. Humulone suppressed the activation of mitogen-activated protein kinases (MAPKs) in TPA-treated mouse skin. The roles of extracellular signal-regulated protein kinase-1/2 and p38 MAPK in TPA-induced activation of NF-κB in mouse skin had been defined in our previous studies. The present study revealed that topical application of SP600125, a pharmacological inhibitor of c-Jun-N-terminal kinase (JNK), abrogated the activation of AP-1 and the expression of COX-2 in TPA-treated mouse skin. Taken together, humulone suppressed TPA-induced activation of NF-κB and AP-1 and subsequent expression of COX-2 by blocking upstream kinases IκK and JNK, respectively, which may account for its antitumor-promoting effects on mouse skin carcinogenesis.

Introduction

Inflammation acts as a driving force in pre-malignant and malignant transformation of cells (1). An improper up-regulation of signal transduction pathways initiated by pro-inflammatory stimuli has been implicated in tumor promotion (2,3). Inappropriate over-expression of cyclooxygenase-2 (COX-2), a rate-limiting enzyme involved in prostaglandin biosynthesis and inflammation, has been frequently observed in various pre-malignant and malignant tissues (4,5). It has been reported that COX-2 over-expressing mice are sensitive (6), whereas COX-2 knockout animals are less prone (7) to chemically induced skin tumor formation. A rapid and transient induction of COX-2 has been observed in cells or tissues exposed to pro-inflammatory and mitogenic stimuli including cytokines, endotoxins, growth factors, lipopolysaccharide, ultraviolet radiation and phorbol ester (8).

Topical application of a prototype tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA) induces the expression of COX-2 and its mRNA transcript in mouse skin (9). As underlying mechanisms of COX-2 induction, the TPA-induced activation of eukaryotic transcription factors, especially nuclear factor-kappaB (NF-κB) (10) and activator protein-1 (AP-1) (11), and upstream kinases, including inhibitory kappaB (IκB) kinase (IKK) (12), extracellular signal-regulated protein kinase (ERK) (9) and p38 mitogen-activated protein kinase (MAPK) (11) has been reported. These intracellular signaling molecules are prime targets of chemoprevention with structurally diverse dietary phytochemicals (3).

Humulone, a bitter ingredient present in hop (Humulus lupulus L.), possesses antioxidant (13), anti-angiogenic (14) and apoptosis-inducing (15) properties. Yasukawa et al. (16) reported that topical application of humulone (1 mg/mouse) significantly inhibited TPA-induced tumor formation in mouse skin stimulated with 7,12-dimethylbenz[a]anthracene. However, the molecular mechanisms underlying the antitumor-promoting effect of humulone have not been elucidated yet. The inhibitory effect of humulone on the transcriptional activation of COX-2 has been demonstrated in cultured cells (17,18). The present study has been aimed to investigate the effects of humulone on TPA-induced COX-2 expression in mouse skin in vivo and to explore underlying molecular mechanisms.

Materials and methods

Materials

Humulone was kindly supplied from Dr Myung Hwan Park (Ambo Institute, Seoul, Korea). The structural identity was confirmed by a series of spectroscopic analyses. TPA was purchased from Alexis Biochemicals (San Diego, CA). Rabbit polyclonal COX-2 antibody was the product of Cayman Chemical Co. (Ann Arbor, MI). Primary antibodies for ERK, p38, p65, p50, IκBα, IKKα and -κB and c-Jun-N-terminal kinase (JNK) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-p38, anti-phospho-JNK and anti-phospho-ERK were obtained from Cell Signaling Technology (Beverly, MA). The anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were obtained from Zymed Laboratories (San Francisco, CA). Oligonucleotide probes containing NF-κB or AP-1 were obtained from Promega (Madison, WI). [γ-32P]ATP was purchased from Amersham Pharmacia Bio-tech (Buckinghamshire, UK). The electrophoretic mobility shift assay kit was obtained from Gibco BRL (Grand Island, NY).

Animal treatment

Female ICR mice (6–7 weeks of age) were supplied from Sankyo Laboservice Corporation (Tokyo, Japan). Animals were housed in climate-controlled quarters (24°C at 50% humidity) with a 12 h light–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. Humulone (1 or 10 nmol) and TPA (10 nmol) were dissolved in 200 μl of acetone and applied topically to the dorsal shaved area.

Western blot analysis

Female ICR mice were topically treated on their shaved backs with indicated doses of humulone 30 min before TPA (10 nmol) treatment and killed by cervical dislocation 1 or 4 h later. Collected tissues were lysed in 800 μl 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 NaVO₄ and protease inhibitor cocktail tablets] and lysates were centrifuged at 14800 g for 15 min. Aliquots of...
supernatant containing 30 μg protein were boiled in 5× sodium dodecyl sulfate (SDS) sample-loading buffer for 5 min before electrophoresis on a 10 or 12% SDS–polyacrylamide gel. After transferring the SDS–polyacrylamide gel to polyvinylidene difluoride membrane, the membranes were blocked with 5% fat-free dry milk in 1× PBST buffer (phosphate-buffered saline containing 0.1% Tween 20) for 2 h at room temperature (RT). The blocked membranes were incubated with 1:4000 dilutions of anti-actin antibody (for 4 h, RT), with 1:1000 dilutions of primary antibodies for COX-2, ERK, pERK, p38 and JNK (for 12 h, 4°C) or with 1:500 dilutions of primary antibodies for IκBα, p65, phospho-IκBα, phospho-p38, phospho-JNK, phospho-p65-(Ser 536) and phospho-p65-(Ser 276) (for 48 h, 4°C). Blots were washed three times with 1× PBST for 5 min each, then incubated with 1:5000 dilutions of anti-rabbit or anti-mouse horseradish peroxidase conjugated-secondary antibodies for 1 h at RT and again washed three times with 1× PBST for 5 min each. The transferred proteins were visualized with an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech) according to the manufacturer’s protocol.

**Immunohistochemical analysis**

The dissected skin was prepared for immunohistochemical analysis of the expression pattern of COX-2 in mouse skin treated with TPA in the presence or absence of humulone (10 μmol). Four-micrometer sections of 10% formalin-fixed, paraffin-embedded tissues were cut in silanized glass slides and deparaffinized three times with xylene and rehydrated through graded alcohol bath. The deparaffinized sections were heated and boiled twice for 6 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval. To diminish non-specific staining, each section was treated with 3% hydrogen peroxide and 4% peptone casein blocking solution for 15 min. For the detection of COX-2, slides were incubated with affinity purified rabbit polyclonal anti-COX-2 antibody (Cayman Chemical Co.) at RT for 40 min in Tris-buffered saline containing 0.05% Tween 20 and then developed by using anti-rabbit HPR EnVisionTM System (Dako, Glostrup, Denmark). The peroxidase-binding sites were detected by staining with 3,3′-diaminobenzidine tetrahydrochloride (Dako). Finally, counterstaining was performed using Mayer’s hematoxylin.

**Fig. 1.** Inhibitory effects of humulone on TPA-induced COX-2 expression in mouse skin. (a) Shaven backs of female ICR mice were treated topically with TPA (10 nmol/0.2 ml acetone) in presence or absence of humulone (1 or 10 nmol/0.2 ml acetone). Control animals were treated with acetone in lieu of TPA. Mice were killed after 4 h. Total cell lysates were analyzed for COX-2 expression by immunoblotting. Quantification of COX-2 immunoblot was normalized to that of actin followed by statistical analysis of relative image density. a, \( P < 0.01 \) (control versus TPA alone); b, \( P < 0.01 \) (TPA alone versus 1 nmol humulone plus TPA); c, \( P < 0.01 \) (TPA alone versus 10 nmol humulone plus TPA). (b) Skin samples from mice treated with acetone (left), TPA alone (center) and humulone (10 nmol) plus TPA (right) were subjected to immunohistochemical analysis by using affinity purified murine COX-2 antibody as described in Materials and methods. Positive COX-2 staining yielded a brown-colored product. (c) Percent of COX-2 positivity in epidermal layer was determined by counting the number of total and COX-2-positive cells from 10 equal sections of immunostained tissues from each animal. a, \( P < 0.01 \) (control versus TPA alone); b, \( P < 0.01 \) (TPA alone versus 10 nmol humulone plus TPA).
Humulone modulation of COX-2 in mouse skin

Preparation of cytosolic and nuclear extracts from mouse skin

The cytoplasmic and nuclear extracts from mouse skin were prepared as described previously (9). In brief, scraped dorsal skin was homogenized in 1 ml of ice-cold hypotonic buffer A [10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM MgCl2, 1 mM DTT, 0.1 mM EDTA, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethylsulfonylfluoride]. After 15 min incubation on ice, 70 μl of 10% Nonidet P-40, centrifuged, resuspended in 150 μl of buffer A [10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM MgCl2, 1 mM DTT, 0.1 mM phenylmethylsulfonylfluoride and 10% glycerol] and centrifuged [50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM MgCl2, 100 mM NaCl, 1 mM DTT, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM phenylmethylsulfonylfluoride and 10% glycerol]. After 15 min incubation on ice, 70 ml of 10% Nonidet P-40, centrifuged, resuspended in 150 ml of buffer A [10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM MgCl2, 1 mM DTT, 0.1 mM phenylmethylsulfonylfluoride and 10% glycerol] and centrifuged for 2 min at 14800 g. The supernatant was collected as the cytosolic fraction. The precipitated nuclei were washed once with 400 μl of buffer A plus 25 μl of 10% Nonidet P-40, centrifuged, resuspended in 150 ml 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 phenylmethylsulfonylfluoride 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 the protein concentrations.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay was performed by using a DNA–protein binding detection kit according to the manufacturer’s protocol (Gibco BRL). Briefly, oligonucleotide probes for NF-κB (5′-GAG GGG ATT CCC TTA-3′) and AP-1 (5′-CCG TTT ATG AGT CAG CCG GAA C-3′) were separately labeled with [γ-32P]ATP by T4 polynucleotide kinase and purified on a Nick column (Amersham Pharmacia Biotech). The binding reaction was carried out in 25 μl of the mixture containing 5 μl 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 μl of nuclear extracts and 100 000 c.p.m. of [γ-32P]ATP-end-labeled oligonucleotides. After 50 min incubation at RT, 2 μl of 0.1% bronopol blue was added, and samples were electrophoresed through 6% non-denatured polyacrylamide gel at 150 V in a cold room for 2 h. Finally, the gel was dried and exposed to X-ray film.

In vitro IKK activity assay

Cytosolic extracts prepared from mouse skin treated as described in the figure legends were used to measure the IKK activity according to the protocol described previously (12). Briefly, the cytosolic extract (200 μg) was pre-cleared using normal mouse immunoglobulin G and protein G agarose beads. Pre-cleared extract was subjected to immunoprecipitation by using anti-IKKβ and anti-IKKα antibodies, and immunoprecipitates were suspended in 50 μl of a reaction mix containing 48 μl of kinase buffer [25 mM Tris–HCl (pH 7.5), 5 mM glycerolphosphate, 2 mM DTT, 0.1 mM Na2VO4 and 10 mM MgCl2], 1 μg GST-IκBα substrate protein and 10 μCi [γ-32P]ATP and incubated at 30°C for 45 min. The kinase reaction was stopped by adding 15 μl 2.5× SDS loading dye, boiled at 99°C for 5 min, vortexed and centrifuged at 5000 r.p.m. for 2 min. The supernatant was separated by 12% SDS–polyacrylamide gel. The gel was stained with Coomassie brilliant blue and destained with destaining solution (glacial acetic acid:methanol:distilled water, 1:4:5, v/v). The de-stained gel was dried at 80°C for 1 h and was exposed to X-ray film to detect the phosphorylated glutathione S-transferase (GST)-IκBα in the radiogram.

MAPK activity assay (non-radioactive)

For determining the catalytic activity of ERK1/2, an in vitro kinase assay was carried out by using a non-radioactive p44/42 kinase assay kit (Cell Signaling

Fig. 2. Inhibitory effects of humulone on TPA-induced activation of NF-κB in mouse skin in vivo. Shaven backs of female ICR mice were treated either with acetone or humulone (1 or 10 μmol) 30 min prior to TPA (10 nmol) except control animals, which were treated with acetone only. One hour after TPA treatment, the epidermal cytosolic and nuclear extracts were prepared. (a) Inhibitory effect of humulone on TPA-induced NF-κB DNA binding. Data presented are representative of three independent experiments showing a similar trend. (b) Nuclear protein (60 μg) was separated by 10% SDS–polyacrylamide gel and immunoblot was performed by using a primary antibody specific to detect p65 and p50 protein levels. Quantification of p65 immunoblot was normalized to that of actin followed by statistical analysis of relative image density. a, P < 0.01 (control versus TPA alone); b, P < 0.05 (TPA alone versus 1 μmol humulone plus TPA); c, P < 0.01 (TPA alone versus 10 μmol humulone plus TPA). (c) The expression of phosphorylated p65-(serine-536) p65-(serine-276) was measured by immunoblot analysis of cytosolic proteins (60 μg) after separation over 10% SDS–polyacrylamide gel. Data are representative of two independent experiments showing a similar trend. (d) Cytosolic extracts from mice treated with acetone, TPA alone and humulone (1 or 10 μmol) plus TPA were subjected to western blot analysis to examine the expression of pIκBα and IκBα using specific antibodies. Quantification of band intensity of pIκBα and subsequent statistical analysis were performed. a, P < 0.01 (control versus TPA alone); b, P < 0.01 (TPA alone versus 1 μmol humulone plus TPA); c, P < 0.01 (TPA alone versus 10 μmol humulone plus TPA).
Technology) following the manufacturer’s protocol. Collected tissues were lysed in 200 µl of lysis buffer per sample [20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 mM Na3VO4, 1 g/ml leupeptin]. The lysate was centrifuged, and the supernatant was incubated with specific immobilized phospho-ERK1/2 monoclonal antibody with gentle rocking for overnight at 4°C. The beads were washed twice each with 500 µl of lysis buffer and the same volume of kinase buffer [25 mM Tris–HCl (pH 7.5), 5 mM glycerophosphate, 2 mM DTT, 0.1 mM Na3VO4, and 10 mM MgCl2]. The kinase reactions were carried out in the presence of 100 µM ATP and 2 µg of Elk fusion protein at 30°C for 30 min. The phosphorylation of Elk was selectively measured by immunoblotting with a specific antibody detecting phospho-Elk.

Statistical analysis

Data were analyzed for main effects by one-way analysis of variance. When the main effect was significant, the Dunnett’s post hoc test was applied to determine individual differences between means. A value of $P < 0.05$ was considered significant. Statistical analysis was performed using Graph Pad software (GraphPad Software).

Results

Humulone suppressed TPA-induced COX-2 expression in mouse skin

We have reported previously that the topical application of TPA (10 nmol) on to shaven backs of female ICR mice induced COX-2 protein expression maximally at 4 h (9). In the present study, we observed that pre-treatment of mouse skin with humulone (10 µmol) significantly inhibited TPA-induced COX-2 expression (Figure 1a). Immunohistochemical analysis of sections of mouse skin treated with TPA verified the inhibitory effect of humulone on TPA-induced epidermal COX-2 expression (Figure 1b). As shown in Figure 1c, pre-treatment with humulone significantly inhibited the number of epidermal COX-2-positive cells in comparison with TPA treatment alone.

Humulone suppressed TPA-induced activation of NF-κB in mouse skin

In response to pro-inflammatory or oxidative stimuli, the up-regulation of COX-2 expression requires the activation of several transcription factors including NF-κB and AP-1 (9,11,19). Therefore, we first examined the effects of humulone on TPA-induced activation of NF-κB in mouse skin. Pre-treatment with humulone suppressed TPA-induced NF-κB DNA binding (Figure 2a). In addition, TPA-stimulated nuclear translocation of p65 and p50 subunit proteins of NF-κB (Figure 2b) was attenuated by pre-treatment with humulone.

In another experiment, pre-treatment with humulone significantly diminished TPA-stimulated phosphorylation and subsequent degradation of IkBα (Figure 2d).

Inhibitory effects of humulone on IKK activity in TPA-treated mouse skin

It has been demonstrated that the activation of NF-κB and the induction of COX-2 in TPA-stimulated mouse skin is mediated via the activation of upstream kinase IKK (12). In the present study, we noted that TPA induced IKKβ activity to a greater extent than IKKα activity and humulone significantly inhibited both IKKα and IKKβ activities in TPA-stimulated mouse skin (Figure 3a). The IKKβ activity was suppressed to the greater extent than the IKKα activity, and humulone significantly diminished TPA-stimulated phosphorylation and subsequent degradation of IkBα (Figure 3d).
Humulone modulation of COX-2 in mouse skin

**Discussion**

Despite a substantial progress in developing new anticancer therapies, cancer is still a global health problem. While the mortality from cancer is increasing worldwide, glimpses of hope flare through recent studies supporting that cancer could be one of preventable diseases. Chemoprevention, which refers to the use of non-toxic substances from natural or synthetic origin to reverse or block carcinogenesis, is a rational approach to prevent cancer (3,22). A wide variety of dietary phytochemicals have been shown to interfere with initiation, promotion and progression stages of carcinogenesis (3). Although diverse mechanisms have been accounted for the chemopreventive effects of dietary phytochemicals (3,22), attention has recently been focused on intracellular signaling cascades as common molecular targets of various chemopreventive phytochemicals (2,23). Because of a causal relationship between inflammation and cancer (24,25), targeted blockade of intracellular signaling pathways mediating inflammatory response is now considered as a road map for developing molecular target-based chemopreventive agents (20). Multiple lines of evidence suggest that the modulation of cellular signaling network involved in aberrant COX-2 induction is a pragmatic approach for the elucidation of molecular basis of chemoprevention by dietary phytochemicals (26,27).

Humulone, a bitter acid from hop, has been reported to suppress TPA-promoted tumor formation in 7,12-dimethylbenz[a]anthracene-initiated mouse skin (16). However, the underlying molecular mechanisms of antitumor-promoting activity of humulone still remain to be elucidated. The present study has been designed to examine the effects of humulone on TPA-induced activation of intracellular signaling molecules, which are involved in aberrant COX-2 expression in mouse skin. Although humulone has been reported to inhibit tumor necrosis factor (TNF-α)-induced COX-2 expression in vitro (17), our study appears to be the first in vivo evidence of the inhibitory effect of humulone (10 μmol) on COX-2 expression in TPA-treated mouse skin.

The induction of COX-2 in TPA-stimulated mouse skin is regulated by a variety of transcription factors including NF-κB and AP-1 (9,11,28). In resting cells, NF-κB resides in cytoplasm by forming an inactive complex with inhibitory protein IκBα. Upon exposure to pro-inflammatory stimuli, IκBα gets phosphorylated and subsequently

antibody. Thereafter, it was incubated with humulone (100 μM) dissolved in dimethyl sulfoxide or vehicle for 30 min, and the kinase assay was performed. Humulone directly inhibited the IKKβ activity in vitro (Figure 3b) as it did when topically applied on mouse skin (Figure 3a).

**TPA-induced activation of AP-1 in mouse skin was inhibited by humulone**

AP-1, another ubiquitous eukaryotic transcription factor, has been attributed to the elevated expression of COX-2 in TPA-stimulated mouse skin (11). Pre-treatment with humulone strongly inhibited TPA-induced AP-1 DNA binding (Figure 4a). Western blot analysis showed that nuclear expression of c-Jun and c-Fos in TPA-treated mouse skin was diminished by pre-treatment with humulone (Figure 4b).

**Humulone inhibited the activation of MAPKs in mouse skin stimulated with TPA**

MAPKs are known to regulate COX-2 expression by diverse mechanisms including the modulation of signaling via both NF-κB and AP-1 (20). Previous studies from this laboratory have reported that TPA-induced activation of ERK and p38 MAPK regulates COX-2 expression in mouse skin in vivo (9,11,21). We, therefore, examined the effect of humulone on TPA-induced phosphorylation of MAPKs by western blot analysis. As illustrated in Figure 5a, humulone blunted the phosphorylation of ERK1/2, p38 MAPK and JNK in TPA-treated mouse skin in a dose-dependent manner. An in vitro kinase assay revealed that humulone inhibited the catalytic activity of ERK1/2 as evidenced by a significant decrease in the expression of phospho-Elk protein (Figure 5b).

**SP600125, a specific inhibitor of JNK, blunted TPA-induced activation of AP-1 and expression of COX-2 in mouse skin in vivo**

Roles of ERK1/2 and p38 MAPK in regulating TPA-induced COX-2 expression in mouse skin have been reported earlier from this laboratory (9,11,21). We have also reported that p38 MAPK, but not ERK1/2, inhibited TPA-induced activation of AP-1 in mouse skin (11). Since humulone inhibited phosphorylation of JNK, we attempted to examine whether JNK also plays a critical role in regulating TPA-induced AP-1 activation and COX-2 expression in mouse skin in vivo. Topical application of SP600125 (1 or 4 μmol), a pharmacological inhibitor of JNK, suppressed TPA-induced phosphorylation of JNK (Figure 6a), DNA binding of AP-1 (Figure 6b) and the expression of c-Jun and c-Fos proteins (Figure 6c). In addition, SP600125 significantly attenuated TPA-induced expression of COX-2 in mouse skin (Figure 6d). These findings suggest that TPA-induced COX-2 expression in mouse skin is partly mediated via JNK–AP-1 signaling pathway.
degraded by proteasomal system, thereby leaving NF-κB free to translocate to the nucleus (29,30). Once in the nucleus, NF-κB regulates transcription of *cox-2* by binding to the consensus NF-κB binding sequence located in the promoter region of *cox-2* gene (9,31). The present study has revealed that humulone abolished both DNA binding and nuclear translocation of NF-κB in TPA-stimulated mouse skin by blocking phosphorylation and subsequent degradation of IκBα. The efficient transcriptional activation of NF-κB depends on the phosphorylation of its active subunit p65/RelA (32). The inhibitory effect of humulone on TPA-induced phosphorylation of p65/RelA on serine-536 and serine-276 residues, therefore, indicates that humulone can attenuate NF-κB transactivation in mouse skin *in vivo*. Yamamoto *et al.* (17) also demonstrated that humulone suppressed TNF-α-induced NF-κB transcriptional activity in MC3T3-E1 cells, an osteogenic cell cloned from newborn mouse clavaria. The effect of humulone on the NF-κB-luciferase reporter gene assay *in vivo* merits further investigation.

In a recent study, we have reported that topical application of Bay 11-7082, a pharmacological inhibitor of IKK, attenuated TPA-induced activation of NF-κB and expression of COX-2 in mouse skin (12). The IKK has been reported to regulate the phosphorylation of both IκBα and p65 (33,34). Within the IKK complex, IKKα is largely responsible for p65 phosphorylation, whereas IKKβ is capable of phosphorylating both IκBα and p65 (35). The present study revealed that humulone significantly inhibited TPA-induced IKKβ activity and to a lesser extent IKKα activity in mouse skin.

Besides NF-κB, AP-1 regulates the transcription of a vast variety of genes, some of which are involved in neoplastic transformation and tumor promotion (36,37). It has been demonstrated that TPA-induced expression of COX-2 in mouse skin is regulated partly by AP-1 (11). Considering the importance of AP-1 in tumor promotion, the inhibition of DNA binding of this transcription factor and expression of its components partly explains the molecular basis of the antitumor-promoting effect of humulone on mouse skin carcinogenesis.

We have also reported that MAPKs play a critical role in the transcriptional activation of NF-κB and AP-1 in TPA-treated mouse skin (9,11,21). Among the MAPKs, ERK1/2 and p38 MAPK have been shown to regulate activation of NF-κB by diverse mechanisms involving enhanced phosphorylation of IκBα and p65 (9,21). Moreover, we have reported that p38 MAPK regulates the induction of AP-1 in mouse skin stimulated with TPA (11). The inhibitory effects of humulone on TPA-induced phosphorylation of ERK1/2 and p38 MAPK, therefore, suggest a molecular basis of the inhibitory effects of humulone on TPA-induced activation of NF-κB and AP-1.

Several studies have demonstrated the role of JNK in mediating AP-1 activation and COX-2 expression in various cultured cell lines (38,39). We have attempted to explore the role of JNK in TPA-induced activation of AP-1 and expression of COX-2 in mouse skin *in vivo*. The inhibition of TPA-induced DNA binding of AP-1, the expression of c-Jun and c-Fos proteins and the expression of COX-2 in mouse skin by pre-treatment with SP600125 suggests that JNK is involved in the TPA-induced activation of AP-1 and expression of...
COX-2 in mouse skin. Therefore, the inhibitory effects of humulone on the AP-1 activation and COX-2 expression in TPA-treated mouse skin may be partly mediated via down-regulation of JNK phosphorylation. Recently, ERK5 has also been reported to play a critical role in regulating normal physiological functions, such as survival, proliferation and differentiation, as well as in carcinogenesis and other pathological processes (40,41). It has been demonstrated that the activation of ERK5 is involved in gastrin-induced COX-2 expression in intestinal epithelial cells (40). The possible induction of ERK5 in TPA-treated mouse skin and its modulation by humulone cannot be excluded.

In conclusion, humulone inhibited TPA-induced COX-2 expression via modulation of NF-κB and AP-1 signaling pathways, which may provide a mechanistic basis of anti-inflammatory and antitumor-promoting activity of humulone in mouse skin in vivo.

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Conflicts of Interest Statement: None declared.

References


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