Caffeic acid suppresses UVB radiation-induced expression of interleukin-10 and activation of mitogen-activated protein kinases in mouse

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Ultraviolet B (UVB) radiation present in sunlight causes sustained immune suppression, photocarcinogenesis and photoaging in humans. Interleukin-10 (IL-10) plays a critical role in UVB-induced immune suppression by inhibiting cell-mediated immune reactions. Mitogen-activated protein kinases (MAPKs) have been implicated in UVB-induced skin carcinogenesis. Caffeic acid (CA), a phenolic acid present in many dietary plants has been shown to confer antioxidant, anti-inflammatory and anticancer activities. In this study, we evaluated the protective effects of CA against UVB radiation-induced IL-10 expression and phosphorylation of MAPKs in mouse skin. An in vivo transgenic IL-10 promoter–luciferase-reporter gene based assay revealed that CA inhibits the transcriptional activation of UVB-induced IL-10 promoter. This was further confirmed by significant inhibition of UVB radiation-induced IL-10 mRNA expression and protein production by CA in mouse skin. Contact hypersensitivity assay showed that CA could attenuate the local immune suppression induced by UVB radiation against a hapten, dinitrofluorobenzene. Our results indicated that CA might inhibit IL-10 production by interfering with an early step, prostaglandin E₂ synthesis, in the activation of UVB-induced immune suppressive cytokine cascade. CA also significantly inhibited the UVB-induced activation of MAPK signal transduction pathways, such as extracellular signal-regulated protein kinase, c-Jun N-terminal protein kinase and p38 mitogen-activated protein kinase, and the downstream transcription factors activator protein-1 and nuclear factor-kappa B. The findings of our study suggest that CA may confer significant protection against UVB-induced immune suppression and photocarcinogenesis in vivo and provide the possible underlying molecular basis for its actions. Therefore, CA may have therapeutic potential as a topical protective agent against the deleterious effects of UVB radiation.

Introduction

The UVB (280–320 nm) component of solar ultraviolet radiation has been shown to suppress the immune system and act as a tumor initiator, tumor promoter and co-carcinogen (1–3). Immunological studies with skin cancer patients have indicated that the immune suppression induced by UVB radiation is a major risk factor in skin cancer development (4). In addition to suppressing tumor rejection, UVB radiation interferes with a wide variety of immune reactions including contact hypersensitivity (CHS) to chemical allergens, and delayed-type hypersensitivity (DTH) to bacterial and viral antigens (5–7). Experimental evidence suggests that the production of immunomodulatory cytokines such as interleukin-10 (IL-10) from keratinocytes and other target cells in the skin plays a crucial role in UVB radiation-induced immune suppression. IL-10 has been shown to play a key role in UVB-mediated systemic suppression of DTH (8). Intraperitoneal injection of IL-10 into mice has been shown to suppress the effector phase of CHS and the induction phase of DTH (9). Further, resistance of IL-10 deficient mice to UVB-induced suppression of DTH substantiated the key role of this cytokine (10). Neutralizing anti-IL-10 antibodies reversed the systemic immune deficit created by acute, low-dose UVB radiation (11).

There is considerable evidence that the tumor-promoting effects of UVB radiation are mediated by the activation of signal transduction pathways that control gene expression (12). Mitogen-activated protein kinases (MAPKs) belonging to a family of serine/threonine protein kinases are activated by UVB radiation in vitro and in vivo (13,14), and believed to play a crucial role in UVB-induced skin damages. The MAPK family includes extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinase (JNK) and p38 kinase, which play important roles in the regulation of cellular functions, such as proliferation, differentiation, apoptosis and inflammation (15). In addition to normal cellular functions, MAPKs also play key roles in tumor growth and development. Activation of ERK has been shown to be sufficient for the transformation of mammalian cells and is associated with cancer progression (16,17). JNK activity was shown to be required for the transformation of human lung carcinoma cells and JNK knockout mice showed reduced skin tumorigenesis (18,19). Increased level of p38 activation was observed in breast carcinoma cells and it has been shown to be a critical mediator of oncogenic activity in prostate cancer (20,21). Tumor-promoting effects of MAPK family members are mediated through the activation of downstream transcription factors such as, activator protein-1 (AP-1) and nuclear factor-kappa B (NF-κB) (22).

Therapeutic intervention through blocking UVB radiation-induced expression of immune suppressive molecule IL-10, and the activation of MAPK signal transduction pathways and downstream transcription factors may be beneficial in preventing skin cancer. Regular intake of dietary

Abbreviations: AP-1, activator protein-1; CA, caffeic acid; CHS, contact hypersensitivity; COX-2, cyclooxygenase-2; EGC, epigallocatechin-3-gallate; ERK, extracellular signal-regulated protein kinase; DTH, delayed-type hypersensitivity; HY, hydrocortisone; IL-10, interleukin-10; JNK, c-Jun NH₂-terminal protein kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappa B; p38, p38 mitogen-activated protein kinase; PGE₂, prostaglandin E₂; UVB, ultraviolet B radiation.
antioxidants or treatment of the skin with products containing antioxidant ingredients has been suggested as a useful strategy for the prevention of UV-mediated cutaneous damages (23). Caffeic acid (3,4-dihydroxycinnamic acid, CA) is a common phenolic acid, naturally found in fruits, vegetables, wine, olive oil and coffee (24). CA has been shown to confer a wide spectrum of biological effects such as antioxidant, antitumor, anti-metastatic, anti-inflammatory, antiviral and anti-rheumatic properties (25–29). Furthermore, CA has been shown to afford significant protection against UVB-induced erythema in humans and UVC-induced cytotoxicity in cell lines (30,31). However, so far the mechanistic bases for its various therapeutic effects have not been reported.

The above research findings warrant a scientific investigation into the possible potential use of CA as a protective agent against UVB radiation-induced skin damages. The purpose of this study is to investigate the immunoprotective and chemopreventive properties of topically applied CA by targeting UVB radiation-induced IL-10 expression and activation of MAPK signal transduction pathways in mouse skin.

Materials and methods

Mice
Female BALB/cBYJ mice (National Laboratory Animal Breeding and Research Center, Taipei, Taiwan) were maintained under pathogen-free conditions on standard laboratory chow and water in the animal facilities of the Institute of Biological Chemistry, Academia Sinica. All mice used in our experiments were 7–8 weeks old.

Phytochemicals and chemicals
CA was purchased from Calbiochem (La Jolla, USA) and its chemical structure and molecular weight are shown in Figure 1. EGCG ([1−]-epigallocatechin-3-gallate) and hydrocortisone (HY; 11β, 17α, 21-trihydroxyprog-4-ene-3, 20-dione) were purchased from Sigma (St Louis, MO) and used as positive reference controls. Stock solutions of the above compounds were prepared in a solvent containing 95% acetone and 5% dimethyl sulfoxide (DMSO) at high concentrations and diluted in acetone prior to use. DNFB (2, 4-dinitrofluorbenzene) was obtained from Sigma and dissolved in acetone.

Plasmid construction, in vivo transfection and luciferase assay
Plasmid pLL-10P-Luc was constructed by isolating promoter region (~1343/+26 bp) of human IL-10 flanked by MluI and BglII restriction sites from genomic DNA through PCR and cloned into the pGL3-Basic vector (Promega, Madison, WI). Plasmid construct was verified by DNA sequencing. The promoterless pGL3-Basic vector was used as a negative control plasmid for luciferase assays. Plasmids pAP-1-Luc and pNF-kB-Luc containing seven AP-1 and five NF-kB binding sites, respectively, along with a basic promoter element (TATA) driving luciferase reporter gene were purchased from Stratagene (La Jolla, CA). Plasmid DNA used in transient transfection assays was isolated using an endotoxin-free mega plasmid purification kit (QIAGEN GmbH, Germany). In vivo particle mediated gene transfer and luciferase assays were performed as described in our previous studies (32). Briefly, mice were shaved on the abdominal area with electric clippers followed by the application of Nair depilatory lotion at least 24 h before the commencement of the experiment. The target skin area was bombarded once to deliver 1.25 μg of plasmid DNA coated onto 0.5 mg of gold particles with a 380 p.s.i. (pounds per square inch) helium gas pressure. Skin samples were prepared in 500 μl of lysis buffer (1× PBS, 0.1% Triton X-100 and protease inhibitors). Luciferase activity (Promega) was analyzed with a Lumat L9507 luminometer (Berthold, Germany).

UVB irradiation
The source of UVB radiation was UV109 (Waldmann, Medizintechnik, Germany) equipped with a single UV compact fluorescent lamp (narrow band UVB, TL01). One hundred percent energy emission was at wavelength 312 nm with a radiation intensity of 1.06 mW/cm² at a lamp to target distance of 10 cm as measured by a Variocontrol Digital Radiometer (Waldmann, Medizintechnik, Germany). All the experimental mice were shaved on the abdominal area with electric clippers followed by the application of Nair depilatory lotion at least 24 h before the commencement of the experiment. Mice were anesthetized by ketamine hydrochloride injection (0.5 mg/mouse) and a restricted area of abdominal skin was exposed to single or multiple doses of UVB radiation (4000 J/m²) as required. The dose of UVB radiation used in this study was sub-erythemogenic to the experimental mice.

CHS assay
Normal experimental mice were un-irradiated, UVB-irradiated or UVB-irradiated plus test agent treated for two consecutive days. Mice were sensitized on UVB-irradiated abdominal skin by topical application of 50 μl of 0.5% DNFB dissolved in acetone. Five days after sensitization, both dorsal and ventral surfaces of mouse ears were challenged each with 20 μl of 0.2% DNFB. Ear thickness was measured immediately before and after 48 h after the challenge using dial calipers equipped with a micrometer (Mitutoyo Corporation, Japan) and was expressed in ×10⁻۷ mm. DNFB sensitized and challenged mice were used as positive controls. Mice that were not sensitized but challenged with DNFB were used as negative controls. Both positive and negative control mice were un-irradiated.

RT–PCR analysis
The expression of endogenous IL-10 mRNA in mouse skin tissues was analyzed by reverse transcription–polymerase chain reaction (RT–PCR). Normal experimental mice were un-irradiated, UVB-irradiated or UVB-irradiated plus treated with test agents or solvent alone. Samples were collected at indicated time points. Frozen skin samples were homogenized in liquid nitrogen. Total RNA was extracted by using TRIzol reagent (Invitrogen Life Technologies, CA) and resuspended in 25 μl of diethyl pyrocarbonate treated water. RT–PCRs were carried out using the AccessQuick RT–PCR system (Promega) according to the manufacturer’s instructions. Briefly, 1 μg of total RNA from each sample was added to a reaction mixture containing 1× AccessQuick master mix (25 μl DNA polymerase, AMV/RT reaction buffer, 25 mM MgSO₄ and 10 mM dNTP mixture), 10 μM each of specific sense and anti-sense primers, 5 U AMV reverse transcriptase and nuclease-free water, to obtain a final volume of 50 μl. Reactions were incubated at 48°C for 60 min and PCR amplification was carried out after denaturing at 95°C for 2 min. The primers contained the following sequences: Mouse IL-10 sense primer 5’-CTGCTATGCTGCCTGCTCTTAC-3’ and anti-sense primer 5’-GTAGACACCTGGTCTTGGAGCC-3’, mouse GAPDH sense primer 5’-CATCACGCCAACCAGAAGCTTGGA-3’ and anti-sense primer 5’-TACTCTTGGAGCCATGAGCCATG-3’. The PCR products were separated on 1.5% agarose gels (Ultrapure, Gibco BRL, NY) along with a molecular weight marker and visualized by UV illumination after staining with 0.5 μg/ml ethidium bromide solution. Gel images were scanned and densitometry analysis of the captured image was performed using BIO-1D image analysis software. The signal intensities of the test gene in different samples were normalized to the respective mouse GAPDH signal intensity.

Enzyme-linked immunosorbent assay (ELISA) for mouse IL-10
Endogenous mouse IL-10 cytokine concentration in mouse serum was determined using Cytosets mouse IL-10 ELISA kit (Biosource International, CA). Normal mice were un-irradiated, UVB-irradiated or UVB-irradiated plus treated with test agents or solvent alone. Serum samples were collected at indicated time points. Briefly, blood samples were allowed to clot for 30 min at room temperature and centrifuged for 10 min at 1000 g. Plates were coated with 100 μl per well of anti-mouse IL-10 coating antibody (1:1000) for 18 h at 4°C and blocked with blocking buffer for 2 h at room temperature. Aliquots of 100 μl of each respective standard, samples and controls were added, and plates were incubated for 1 h and 30 min at room temperature. After four washes, 100 μl of biotinylated anti-mouse IL-10 monoclonal antibody (1:3000) was added and incubated for 1 h at room temperature. Streptavidin–horseradish peroxidase (HRP) (1:5:10000) was added after washing and incubated
for 45 min. After a final wash, TMB (tetramethylbenzidine) substrate solution was added and incubated for 30 min at room temperature in the dark. Absorbance was read at 450 nm in an ELISA reader after the addition of stop solution. The minimum detectable dose of IL-10 was 5 pg/ml.

**Western blot analysis**

Normal mice were un-irradiated, UVB-irradiated or UVB-irradiated plus treated with test agents or solvent alone. Skin samples were collected at indicated time points for isolation of total protein. Excised mouse skin was immediately placed in liquid nitrogen and pulverized in a mortar. The pulverized skin was 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 Na3VO4, and protease inhibitor cocktail tablets) for 10 min and centrifuged at 20,000 g for 20 min. Supernatant containing 50 μg protein was boiled in sodium dodecyl sulfate (SDS) sample loading buffer for 10 min before electrophoresis on 12% NuPAGE Bis–Tris gel (Invitrogen Life Technologies). After electrophoresis for 2 h, proteins in gel were transferred to PVDF membrane (Novex, San Diego, CA), 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 overnight at 4°C with 1:1000 dilution of Phospho-p44/p42 MAPK polyclonal antibody or Phospho-p38 MAPK or Phospho-SAPK/JNK monoclonal antibodies (Cell Signaling Technology, Beverly, MA) or cyclooxygenase-2 (COX-2) polyclonal antibodies (Cayman Chemical, Ann Arbor, MI). Equal protein loading was assessed using mouse β-actin (Sigma Chemical). The blots were rinsed three times with PBST buffer for 5 min each. Washed blots were incubated with 1:2000 dilution of the HRP conjugated-secondary antibody and then washed again three times with PBST buffer. The transferred proteins were visualized with an enhanced chemiluminescence (ECL) detection kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

**Statistical analysis**

The results are expressed as mean ± SD. Statistically significant differences were assessed with an unpaired, two-tailed Student’s t-test.

**Results**

**CA inhibits UVB-induced transgenic IL-10 promoter activity in mouse skin**

To determine whether IL-10 promoter–reporter gene based assay system can be used to identify the protective agents against UVB radiation-induced immune suppression, we transfected mouse skin with plasmid pIL-10P-Luc (−1343/+26 bp) by using a gene gun. Transfected skin was un-irradiated or irradiated with UVB at a dose of 4000 J/m2 and luciferase activity was measured at two different time points. Our data showed that UVB radiation induced the IL-10 promoter activity by 4.6-fold at 16 h post-irradiation as compared with un-irradiated control and remained the same even at 24 h post-irradiation (Figure 2A). We chose the 16 h post-irradiation time point to evaluate the effects of test agents on UVB-induced IL-10 gene promoter activity. Plasmid, pIL-10P-Luc, transfected skin was left un-irradiated or immediately irradiated with UVB at a dose of 4000 J/m2. Irradiated skin was left untreated or immediately treated with solvent alone or with test agent CA, a reference control EGCG or a commercial anti-inflammatory corticosteroid, HY, each at a concentration of 1 mg/site/mouse. We observed that CA, EGCG and HY significantly inhibited UVB-induced IL-10 promoter activity by 84.4, 66.3 and 54.2%, respectively, as compared with the untreated control (Figure 2B). To examine whether the inhibitory effect of CA on UVB-induced IL-10 promoter activity was dose-dependent, we treated the transfected and irradiated mouse skin with different concentrations of CA (0.01, 0.05, 0.2, 0.5, 1 and 2 mg/site/mouse), A significant inhibitory effect was observed at a concentration of 0.2 mg/site/mouse as compared with untreated control. However, at a concentration of 1 mg/site/mouse, CA showed the highest significant inhibition. There was no further increase in the effect of CA after increasing the concentration to 2 mg/site/mouse (Figure 2C). The most effective concentration of CA was 1 mg/site/mouse. We therefore chose this concentration to carry out the following experiments.

**CA inhibits UVB-induced IL-10 mRNA expression in mouse skin**

In order to determine the optimum time point for maximum induction of endogenous IL-10 mRNA expression in response to UVB, we carried out a time course experiment. Mouse skin was left un-irradiated or irradiated with UVB at a dose of 4000 J/m2 and skin samples were collected at different time points. The mRNA expression was analyzed by RT–PCR. We observed the highest IL-10 mRNA expression at 24 h after UVB irradiation and this expression was slightly reduced at 48 h post-irradiation (Figure 3A). Mouse skin was irradiated with UVB as described above and immediately treated with test agents CA, EGCG or HY each at a concentration of 1 mg/site/mouse. RT–PCR analysis revealed a significant inhibition of UVB-induced IL-10 mRNA expression by CA (71.2%), EGCG (56.1%) and HY (41.6%) as compared with the untreated control (Figure 3B).

**CA inhibits UVB-induced IL-10 protein production in mouse skin**

To determine whether the earlier observed effects of CA on IL-10 promoter activity and mRNA expression are translatable to protein production, we performed ELISA of serum samples obtained from experimental mice. Mouse skin was left un-irradiated or irradiated with UVB and samples of serum were collected at different time points and analyzed. In serum samples of mouse, maximum protein production was observed at 48 h after UVB irradiation and there was a sharp decline at 72 h post-irradiation (Figure 4A). We chose the 48 h post-irradiation time point to study the effects of test agents on UVB-induced IL-10 protein production. Mouse skin was left un-irradiated or irradiated with UVB and immediately treated with test agents CA, EGCG or HY each at a concentration of 1 mg/site/mouse. After 48 h, serum samples were collected and analyzed for IL-10 protein production. Our results indicated that CA (59.4%) significantly inhibited the UVB-induced IL-10 protein production followed by EGCG (49.9%) and HY (34.5%) in mouse serum (Figure 4B).

**CA prevents UVB-induced local immune suppression**

Previous research has shown an important role for IL-10 in UVB-induced immune responses, such as CHS. The above results indicated that CA significantly inhibited the UVB-induced IL-10 expression. Therefore, we sought to determine whether CA could confer protection against UVB-induced suppression of CHS to a hapten, DNFB, in a local model of immune suppression. For this purpose, the abdominal area of mice was left un-irradiated or irradiated with UVB at a dose of 4000 J/m2. Irradiated skin was immediately treated with test agents CA, EGCG or HY at a concentration of 1 mg/site/mouse. After 48 h, serum samples were collected and analyzed for IL-10 protein production. Our results indicated that CA (59.4%) significantly inhibited the UVB-induced IL-10 protein production followed by EGCG (49.9%) and HY (34.5%) in mouse serum (Figure 4B).

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after UVB irradiation showed significant increase in CHS response (ear swelling) as compared with UVB-irradiated but untreated mice (Figure 5). Treatment with test agents CA, EGCG and HY alone did not show any effect on ear swelling of un-irradiated mice (data not shown).

**CA inhibits UVB-induced COX-2 protein production in mouse skin**

Earlier studies have suggested that UVB radiation-induced prostaglandin E\(_2\) (PGE\(_2\)) plays an important role in the secretion of immune suppressive cytokines, including IL-10. Therefore, we wanted to determine whether CA could inhibit UVB-induced production of COX-2, a key enzyme for the synthesis of inducible PGE\(_2\). For this purpose, we performed western blot analysis of protein samples obtained from experimental mice using mouse COX-2 specific antibodies. We first studied the kinetics of UVB-induced COX-2 protein production in mouse skin, which showed maximum induction at 8 h post-irradiation and remained the same at 12 h, but it gradually decreased over the 48 h period after irradiation (Figure 6A). We chose the 8 h post-irradiation time point to study the effect of test agents on UVB-induced COX-2 protein production. Mouse skin was irradiated with UVB at a dose of 4000 J/m\(^2\) and immediately treated with test agent CA (0.2, 0.5 and 1 mg/site/mouse) or a positive reference control, EGCG (1 mg/site/mouse). Our results showed that both CA and EGCG significantly inhibited the UVB-induced COX-2 protein production at the concentration of 1 mg/site/mouse as compared with the untreated control (Figure 6B).

**CA inhibits UVB-induced phosphorylation of MAPKs in mouse skin**

Many previous studies have shown that UVB radiation induces the activation of MAPKs, such as ERK1/2, JNK and p38, which have been implicated in the promotion of skin carcinogenesis. In order to investigate the chemopreventive effects of CA, we studied its effects on UVB-induced phosphorylation of the three MAPKs through western blot analysis using phospho-specific antibodies; mentioned earlier. The data on the kinetics of MAPK activation from UVB-irradiated mouse skin showed that maximum phosphorylation of ERK1/2 (p42/p44) was at 30 min post-irradiation (Figure 7A) and
remained the same at 1 h, but it gradually decreased and reached the control level at 48 h after irradiation (data not shown). Phosphorylation of both JNK (p46/p54) and p38; however, was observed at 4 h, reached a maximum at 8 h (Figure 7B and C) and remained activated even at 48 h after UVB irradiation (data not shown). To determine the effect of CA on UVB-induced activation of MAPKs, mouse skin was irradiated with UVB at a dose of 4000 J/m$^2$ and immediately treated with test agent CA or a reference control (EGCG) or HY each at a concentration of 1 mg/site/mouse. Our results indicated that CA and EGCG significantly inhibited the UVB-induced phosphorylation of ERK1/2 at 30 min after irradiation (Figure 7A). Both CA and EGCG also significantly inhibited UVB-induced phosphorylation of JNK and p38 at 8 h after irradiation (Figure 7B and C). HY did not show any effect on UVB-induced phosphorylation of MAPKs.

**CA inhibits UVB-induced activation of AP-1 and NF-kB transcription factors in mouse skin**

The results mentioned earlier clearly indicate that CA inhibits signal transduction by MAPKs. Therefore, we carried out experiments to determine whether CA could inhibit activation of transcription factors, AP-1 and NF-kB, which are downstream of the MAPK signal transduction pathways. For this

Fig. 3. CA inhibits UVB-induced IL-10 mRNA expression in mouse skin. Mouse skin was left un-irradiated or UVB-irradiated and total RNA was extracted from tissues and analyzed by RT–PCR as described under Materials and methods. (A) Samples were collected at different time points as indicated after UVB irradiation and analyzed for mRNA expression. (B) UVB-irradiated mouse skin was untreated or immediately treated with solvent alone or with CA, EGCG or HY each at a concentration of 1 mg/site/mouse. Samples were collected at 24 h post-irradiation and analyzed for mRNA expression. The effect of test agents on mRNA expression was quantified by densitometry analysis using an image analyzer. The changes in the signal intensity of the test gene relative to GAPDH (housekeeping gene) were calculated and expressed as a percentage of untreated but UVB-irradiated control. These experiments were repeated two times with similar results. m, mouse.

Fig. 4. CA inhibits UVB-induced IL-10 protein production in mouse. (A) Mouse skin was un-irradiated or UVB-irradiated, and serum samples were collected at different time points, processed and analyzed for IL-10 protein production by using specific ELISA kit as described under Materials and methods. (B) UVB-irradiated mouse skin was left untreated or treated with solvent alone or with 1 mg each of CA, EGCG or HY/site/mouse. Forty-eight hours after treatment, serum samples were collected, processed and analyzed as described above. These experiments were repeated three times with similar results. The mean values obtained from three individual mice are shown (± SD). *, $P < 0.05$ and **, $P < 0.01$ versus an untreated but UVB-irradiated control.

Fig. 5. CA prevents UVB-induced local immune suppression. Mouse abdominal skin was un-irradiated or irradiated with UVB. Irradiated skin was then left untreated or immediately treated with CA, EGCG or HY each at a concentration of 1 mg/site/mouse for two consecutive days. Mice were sensitized and challenged by DNFB as described in Materials and methods. CHS response was measured 48 h after challenge and ear thickness was expressed in $\times 10^{-2}$ mm. Un-irradiated mice sensitized and challenged with DNFB served as positive control. Un-irradiated mice not sensitized but only challenged with DNFB served as negative control. This experiment was repeated two times with similar results. The mean values obtained from three individual mice are shown (± SD). *, $P < 0.05$ and **, $P < 0.01$ versus UVB-irradiated mice that were sensitized and challenged.
purpose, we used rapid and quantifiable transgenic-reporter gene based \textit{in vivo} assay system. Mouse skin was transfected with plasmid pAP-1-Luc and left un-irradiated or immediately irradiated with UVB at a dose of 4000 J/m². Transfected and UVB-irradiated skin was immediately treated with solvent alone or test agents CA, EGCG or HY each at a concentration of 1 mg/site/mouse and luciferase activity was measured after 16 h. UVB irradiation significantly increased AP-1 activity by 5.2-fold compared with un-irradiated control. Treatment with CA or EGCG completely abrogated the UVB-induced AP-1 activity, whereas HY treatment did not show any effect (Figure 8). Plasmid pNF-κB-Luc was transfected into mouse skin and transfected skin was treated as described above. Luciferase assay revealed that UVB irradiation significantly increased NF-κB activity by 6.2-fold as compared with un-irradiated control (Figure 9). Treatment with CA and HY significantly decreased UVB-induced NF-κB activation by 72.8 and 66.4%, respectively.

**Discussion**

Several mechanisms have been implicated in UVB-induced abrogation of immune responses; among these modulation of immune regulatory cytokines, such as IL-10, has been suggested to play an important role. Previous studies have shown that UVB irradiation results in increased IL-10 gene expression in the skin (33) and IL-10 protein production in the serum of mice, and treatment with IL-10 antibodies blocks the induction of immune suppression (10). In this study, we also observed significant induction of transgenic IL-10 promoter driven luciferase-reporter gene activity and endogenous IL-10 mRNA expression in mouse skin, and increased IL-10 protein levels in the serum of the mice irradiated with UVB. CA significantly inhibited the UVB radiation-induced transcriptional activity of transgenic IL-10 promoter, endogenous IL-10 mRNA expression and IL-10 protein production indicating its potential to abrogate UVB-induced immune suppression. Many

![Fig. 6. CA inhibits UVB-induced COX-2 protein production in mouse skin.](image)

(A) Mouse skin was un-irradiated or UVB-irradiated, and skin samples were collected at different time points, processed and analyzed for COX-2 protein production by using western blot analysis as described under Materials and methods. (B) UVB-irradiated mouse skin was left untreated or immediately treated with solvent alone or with CA (0.2, 0.5, 1 mg/site/mouse) or EGCG (1 mg/site/mouse). Skin samples were collected 8 h after treatment, processed and analyzed as described above. These experiments were repeated two times with similar results.

![Fig. 7. CA inhibits UVB-induced phosphorylation of MAPKs.](image)

Mouse skin was un-irradiated, UVB-irradiated or UVB-irradiated and immediately treated with solvent or CA, EGCG or HY each at a concentration of 1 mg/site/mouse. Total protein extracts were prepared from skin samples harvested at required time points and subjected to western blot analysis. (A) Skin samples were collected and processed 30 min after UVB irradiation. Phosphorylation of ERK1/2 was detected by phospho-p44/42 MAPK (Thr202/Tyr204) antibodies. (B) Skin samples were harvested at 8 h post-UVB irradiation and phosphorylation of JNK was detected by phospho-p54/p46 JNK (Thr183/Tyr185) antibodies. (C) Skin samples were harvested 8 h after UVB irradiation and phosphorylation of p38 was detected by phospho-p38 MAPK (Thr180/Tyr182) antibodies. Un-irradiated and UVB-irradiated but untreated mice served as negative and positive controls, respectively. Mouse β-actin served as a control for protein input. These experiments were repeated twice with similar results. P, phospho.

![Fig. 8. CA inhibits UVB-induced activation of AP-1.](image)

Mouse skin was transfected with pAP-1-Luc plasmid DNA by using gene gun. Transfected skin was un-irradiated, UVB-irradiated or UVB-irradiated and immediately treated with solvent alone or with CA, EGCG or HY each at a concentration of 1 mg/site/mouse. Skin samples were collected at 16 h post-irradiation and luciferase activity was measured as described in Materials and methods. Luciferase activity was expressed as a percentage of UVB-irradiated but untreated control mice. This experiment was repeated thrice with similar results. The mean values obtained from three individual mice are shown (±SD). **P < 0.01 versus untreated UVB-irradiated control.
models of cell-mediated immune responses such as CHS and DTH have demonstrated that IL-10 plays a critical role in UVB-mediated immune suppression (9). Our current data clearly show that UVB irradiation inhibited the hapten-induced CHS response and treatment of UVB-irradiated skin with CA attenuated the local immune suppression. Previous reports have shown that IL-10 exerts immune suppressive effects by inhibiting the Ag-specific Th1 cell-proliferation via decreased antigen-presenting cell (APC) function at the site of UV exposure (34) as well as at distant sites (35). IL-10 inhibits the APC function by interfering with the up-regulation of co-stimulatory molecules (36) and suppressing the production of immune stimulatory cytokines (37). Our results suggest that CA may prevent UVB-induced immune suppression by inhibiting IL-10 production, and thereby protecting the normal function of antigen-presenting cells and immune surveillance in the skin.

Earlier studies have reported that UVB-induced DNA damage in the form of cyclobutane pyrimidine dimers (CPDs) triggers the production of immune modulatory cytokine IL-10 and the repair of this damage abrogates UVB-induced IL-10 up-regulation and immune suppression (38). It has been suggested that UVB-induced DNA damage activates keratinocytes to synthesize platelet-activating factor (PAF), which in turn binds to PAF receptors on adjacent cells and drives the transcription of immune suppressive cytokines (39). Previous studies have shown that exposure of the skin to UVB radiation induces prostaglandin E2 production (40), and it has been suggested that PGE2 synthesis is an early step in UVB-induced activation of immune suppressive cytokine cascade (41). The role of PGE2 as an initiator of UV-induced cytokine cascade was demonstrated by the use of a specific COX-2 inhibitor, which completely suppressed the secretion of immune suppressive cytokines, including IL-4 and IL-10. This finding was further supported by the observation that injection of PGE2 into normal mice induced the secretion of IL-10 (41). Consistent with previous findings, in this study UVB irradiation of mouse skin resulted in the induction of COX-2 protein production, a key enzyme in the synthesis of PGE2 (42). Treatment with CA significantly inhibited UVB-induced COX-2 production. Based on these findings, we suggest that CA may inhibit IL-10 production by interfering with an early step in the activation of UVB-induced immune suppressive cytokine cascade.

Besides exerting immuno-suppressive effects, UVB radiation also causes skin cancers by acting as a tumor initiator as well as a tumor promoter. UVB radiation promotes tumor development by activating various intracellular signaling cascades that play a major role in cell growth and proliferation leading to clonal expansion of UVB-initiated cells into skin tumors (43). UVB radiation-activated signal transduction pathways are mediated primarily through MAPKs including ERK1/2, JNK and p38 kinases, which control the activities of various downstream transcription factors implicated in tumor promotion (44). ERK1/2 has been shown to have been strongly activated by tumor promoters and growth factors, and has a critical role in transmitting signals initiated by them (45). In this study, we observed an increased activation of ERK1/2 within 30 min of UVB irradiation and this activation was significantly inhibited by topical application of CA on to irradiated skin. Previous studies have shown that a potent inhibitor of the ERK pathway, BAY 43-9006, exhibited a broad spectrum of antitumor activities in colon, breast and non-small-cell lung cancer xenograft models (46). Several studies have reported that activated JNK results in transcriptional activity of protooncoproteins, which are essential for oncogenic transformation (47). Our results have shown increased phosphorylation of JNK at 8 h after UVB irradiation and CA treatment significantly inhibited this activation. Previously, it has been shown that SP600125, a small molecule inhibitor of JNK activity, suppressed tumor growth, angiogenesis and tumor cell-proliferation (48). Various cellular stresses including UVB radiation activate the p38 MAPK pathway, which may have a role in cell-proliferation and cancer development. Here we showed that UVB radiation resulted in increased phosphorylation of p38 and treatment with CA significantly inhibited the p38 activation. Specific inhibition of p38 MAPK activity by SB203580 has been reported to reduce the growth of transformed follicular lymphoma cells (49). Recently, it has been shown that UVB-induced MAPK signaling is also involved in the activation of matrix metalloproteinases causing degradation of skin tissues and photoaging (50). Therefore, inhibition of UVB-induced MAPKs may have broad implications for the prevention and treatment of photocarcinogenesis and photoaging. In this study, CA inhibited all the three MAPK pathways activated by UVB radiation indicating its potential use as a chemopreventive agent.

Activated MAPK family members translocate to the nucleus and phosphorylate the downstream target transcription factor complexes such as AP-1 and NF-kB, which are involved in malignant transformation and tumor promotion (51,52). Inhibition of the activation of transcription factors, AP-1 and NF-kB, has been shown to block the neoplastic transformation response (53). In this study, we observed the activation of AP-1 and NF-kB in mouse skin irradiated with UVB, which is consistent with previous reports (54). Treatment with CA immediately followed by UVB irradiation abrogated the activation of AP-1 and NF-kB transcription factors in mouse skin. Surprisingly, reference control, EGCG, did not show any effect on UVB-induced activation of NF-kB. EGCG has been reported to exhibit dose-based differential inhibition of NF-kB activation (55); therefore, it is possible that the dose used in this study might not be sufficient to inhibit NF-kB...
activation in vivo. Growth factors induce AP-1 by activating ERK subgroup of MAPKs, whereas pro-inflammatory cytokines and genotoxic stresses enhance AP-1 activity through JNK and p38 MAPK cascades (15). ERK and p38 subgroups of MAPKs have been reported to modulate NF-κB activation (56,57). Therefore, we suggest that a significant suppression of UVB-induced phosphorylation of MAPKs, the upstream signal transducers, by CA might be responsible for its inhibitory effects on the activation of transcription factors AP-1 and NF-κB.

In summary, the results of this investigation clearly demonstrate that topicaly applied CA, a common constituent of the human diet, can confer significant protection against UVB radiation-induced harmful molecular effects on the skin. Our findings suggest that CA may prevent UVB-induced immune suppression by inhibiting IL-10 expression. Whereas, CA findings suggest that CA may prevent UVB-induced immune radiation-induced harmful molecular effects on the skin. Our results demonstrate that topically applied CA, a common constituent of the human diet, can confer significant protection against UVB radiation-induced harmful molecular effects on the skin. Our findings suggest that CA may prevent UVB-induced immune suppression by inhibiting IL-10 expression. Whereas, CA findings suggest that CA may prevent UVB-induced immune radiation-induced harmful molecular effects on the skin.

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