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[6]-Gingerol prevents UVB-induced ROS production and COX-2 expression *in vitro* and *in vivo*

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Abstract

[6]-Gingerol, a naturally occurring plant phenol, is one of the major components of fresh ginger (*Zingiber officinale* Roscoe, *Zingiberaceae*) and has diverse pharmacologic effects. Here, we describe its novel anti-oxidant, anti-apoptotic, and anti-inflammatory activities *in vitro* and *in vivo*. *In vitro*, pre-treatment with [6]-gingerol reduced UVB-induced intracellular reactive oxygen species levels, activation of caspase-3, -8, -9, and Fas expression. It also reduced UVB-induced expression and transactivation of COX-2. Translocation of NF-κB from cytosol to nucleus in HaCaT cells was inhibited by [6]-gingerol via suppression of IκBα phosphorylation (ser-32). Examination by EMSAs and immunohistochemistry showed that topical application of [6]-gingerol (30 μM) prior to UVB irradiation (5 kJ/m^2) of hairless mice, also inhibited the induction of COX-2 mRNA and protein, as well as NF-κB translocation. These results suggest that [6]-gingerol could be an effective therapeutic agent providing protection against UVB-induced skin disorders.

Keywords: *UVB, [6]-gingerol, ROS, cyclooxygenase-2, NF-κB, caspase*

Abbreviations: *ROS, reactive oxygen species; COX-2, cyclooxygenase-2; NF-κB, nuclear factor-kappaB; FADD, Fas-associated death domain; NAC, N-acetyl cysteine; IκB, inhibitor of NF-κB*

Introduction

Ultraviolet (UV) radiation, particularly UVB, accounts for acute skin inflammations such as sunburn reactions that lead to severe edema and blistering of the skin [1] and chronic exposure to UV results in premature skin aging, immuno-suppression, skin cancer and cell death [2]. UVB causes a variety of biological effects, including induction of DNA damage, activation of the cell death receptor Fas (CD95) and formation of reactive oxygen species (ROS) [3]. The main DNA lesions induced are cyclobutane pyrimidine dimers and 6–4 photoproducts [4]. It has been proposed that severe DNA damage is a major cause of apoptosis [5]; death receptor induced by UVB leads to recruitment and activation of FADD, which activates caspase-8, which in turn activates caspase-3 [6].

UVB radiation increases the cellular level of ROS [7] which results in oxidative stress to the cells [8–10] and ROS have been shown to initiate cellular damage leading to apoptosis. In addition, ROS have been suggested to play a role as second-messenger molecules in signaling pathways and in the regulation of gene expression related to the biological effects

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elicited by UVB. NF-κB, a redox-regulated transcription factor [11], is involved in regulating cellular processes such as inflammation and apoptosis [12,13], and is upregulated by UVB *in vivo* and *in vitro* [14–16]. However, the exact source of the ROS produced by UVB, and its contribution to NF-κB activation have not been clearly identified. Recent work has indicated that NADPH oxidase may be a major source of the UVB-induced ROS [17]. ROS are known to play an important role in UVB-induced expression of cyclooxygenase-2 (COX-2). This in turn augments the production of a variety of inflammatory mediators such as prostaglandins and leukotriens [18,19] and may also have an important role in the inflammatory reactions provoked by UV irradiation [20,21] Recently much effort has been devoted to identifying chemopreventive phytochemicals of dietary and medicinal origin [22]. Numerous epidemiological as well as laboratory studies suggest that a diet rich in fruit and vegetables has a protective effect against a variety of cancers and other diseases. Although the exact processes underlying these beneficial effects remain unclear, there is evidence that the biologically active phenolic compounds found in various fruits and vegetables offer some benefits [23–25]. Ginger has been used as a spicy condiment for a long time. In addition, the rhizome and root of ginger have been used in traditional oriental medicine to ameliorate symptoms such as nausea and vomiting associated with pregnancy, inflammation, rheumatic disorders and gastrointestinal discomforts [23], [6]-gingerol (1-[4′-hydroxy-3′-methyoxyphenyl]-5-hydroxy-3-decanone; Figure 1), a naturally occurring plant phenol, is one of the major components of fresh ginger, and exhibits diverse pharmacological effects including antioxidant, anti-inflammatory and anti-cancer activities. It also inhibits TPA (12-*O*-tetradecanoylphorbol-13-acetate)-mediated tumor promotion, induction of ornithine decarboxylase, and production of tumor necrosis factor-α (TNF- α) in mouse skin [26]. In addition, it inhibits epidermal growth factor (EGF)-induced neoplastic transformation in mouse epidermal JB6 cells [27]. However, its biological and biochemical activities related to UVB have not been elucidated.

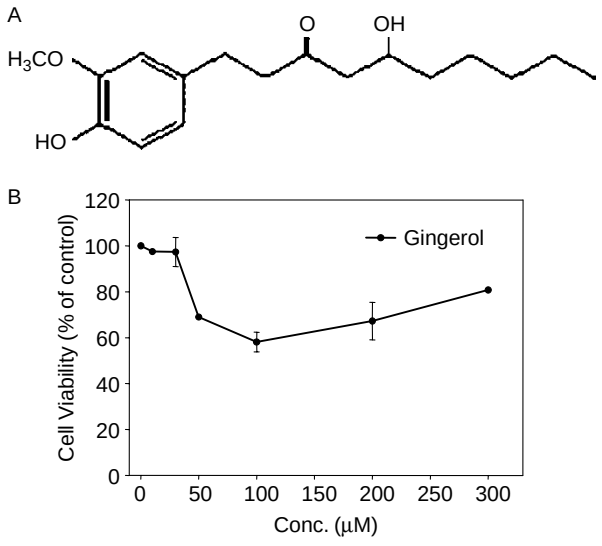

Figure 1. Chemical structure of [6]-gingerol and its effect on cell viablilty. (A) Chemical structure of [6]-gingerol. (B) HaCaT cells were treated with [6]-gingerol in indicated concentration, followed by a MTT assay.

In this study, we determined the effect of [6]-gingerol on UVB-induced COX-2 expression in HaCaT cells and mouse skin. We also investigated the effect of [6]-gingerol on UVB-induced DNA damage. We found that this compound inhibited UVB-induced COX-2 expression and activation of NF-κB by blocking the accumulation of intracellular ROS and prevented UVB-induced caspase activation. These results suggest that it has a photoprotective effect against UVB-induced skin damage.

Materials and methods

Materials

[6]-Gingerol (purity > 98%) was purchased from Wako Pure Chemicals (Osaka, Japan). It was aliquoted in small amounts in DMSO and stored at −20°C. MTT (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide) was obtained from USB Corporation (OH, USA) and Apocynin (Acetovanillone, 98%) and DCFH-DA were from Sigma-Aldrich Chemicals (Germany). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, Utah, USA). COX-2, NF-κB p65, IκBα, and p-IκBα antibodies were products of Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-thymine dimer and anti-actin antibodies were obtained from Sigma (St Louis, MO). The anti-caspase-3, -8, anti-Bcl-2, and anti-Bax antibodies were purchased from Cell Signaling Technology (Beverly, MA) and anti-caspase-9 and anti-Fas antibodies from Upstate (NY). Trizol reagent were purchased from Invitrogen (New Zealand). The Gel Shift Assay System was purchased from Promega Corporation (Madison, WI, USA). [α-^{32}P]dCTP and [γ-^{32}P]ATP were from NEN (Boston, MA, USA).

Cell culture and UVB irradiation of HaCaT cells

The immortalized human keratinocyte cell line, HaCaT, was kindly provided by Professor N. Fusenig (German Cancer Research, Germany) and was grown as monolayer cultures in DMEM supplemented with 10% FBS, 100 units/ml penicillin and 100 μg/ml streptomycin at 37°C under a humidified 95–5% (v/v)

mixture of air and CO_2. The cells were then seeded at 1×10^6 cells/100 mm plates. After 24 h, they were washed with serum-free medium, and replaced in medium without FBS for 12 h prior to treatment with [6]-gingerol.

The serum-starved monolayer cultures were irradiated with a bank of two FS20 Lamps (National Biological, Twinsburg, OH) that emit a continuous spectrum from 270 to 390 nm, with peak emission at 313 nm; approximately 65% of the radiation was within the UVB (280–320 nm) range. The irradiance of a single bulb averaged 6.25 J/m^2/s at a tube-to-target distance of 8 cm, as measured with a spectroradiometer (model IL1700A; International Light, Inc., Newburyport, MA, USA). The HaCaT cells were irradiated with the UVB source at 100 J/m^2 in a minimum amount of PBS (about 3 ml) after washing three times with PBS. Immediately after irradiation, the cells were cultured in serum-free medium for the indicated times with or without addition of drugs.

UVB irradiation of animals

Eight to ten week-old adult male hairless mice (HRS/J hr$^{+/+}$) were obtained from the Korea Research Institute of Biotechnology (Taejeon, Korea). The animals were housed in climate-controlled quarters (24°C at 50% humidity) with a 12 h light/12 h dark cycle. For UVB irradiation, they were placed in a standard cage under six UVB lamps (FS24T12/UVB/HO, 290–320 nm, Voltare Co., Fairfield, CT, USA). These emitted 0.6 mW/cm^2/s, and 5 kJ/m^2 of UVB irradiation were applied. UVB dosage was measured using the spectroradiometer. The [6]-gingerol was dissolved in 200 μl of acetone and applied topically to the back of each mouse.

Preparation of total RNA and Northern blot hybridization

Total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. About 10 μg of total RNA was electrophoresed on a 1.2% agarose gel containing 2.2 mol/l of formaldehyde, transferred to a nylon membrane (Osmonics, Westborough, MA, USA), and covalently linked with an UV cross-linker (UV Stratalinker 1800; Stratagene, La Jolla, CA, USA). Hybridization was performed with [α-^{32}P]dCTP-labeled COX-2 and GAPDH cDNA probes prepared with random primed oligonucleotides (Rediprime DNA Labeling System; Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membrane was washed in 2 × SSC/0.1% SDS at room temperature for 30 min, and 0.1 × SSC/0.1% SDS at 65°C for 30 min. Blots were autoradiographed on X-ray film at −70°C.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from mouse skin using TRIzol Reagent. After DNase treatment, first-strand cDNA synthesis and PCR amplification were carried out using a Reverse Transcription System (Promega Corporation) following the protocol recommended by the manufacturer. Briefly, 1 μg of total RNA were subjected to reverse transcriptase-polymerase chain reaction (RT-PCR) assay, and the correlation between the amounts of RNA used and those of PCR products from both target (COX-2) and internal standard (GAPDH) mRNAs was examined. An aliquot of the cDNA mixture was used for enzymatic amplification. The amplification was performed in a DNA thermal cycler (model PTC-225; MJ Research) under the following condition: denaturation at 94°C for 5 min for the first cycle and for 30 s starting from the second cycle, annealing at 65°C for 1 min, and extension at 72°C for 1.5 min for 30 repetitive cycles. Final extension was at 72°C for 10 min. The primers used were 5′-AAAACCGTGGGGAATGTATGAGC-3′ (sense) and 5′-GATGGGTGAAGTGCTGGGC-AAAG-3′ (antisense) for the COX-2. The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide.

Western blot analysis

Cells were harvested and lysed with RIPA buffer containing 2 mM EDTA, 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM sodium vanadate, 10 mM NaF, 1 mM PMSF, 1% Triton X-100, 10% glycerol and a protease inhibitor cocktail. The protein concentration of each sample was determined using a BCA protein assay kit (Pierce, Rockford, Illinois). Proteins were electrophoresed on SDS-PAGE gels, and transferred to polyvinylidene fluoride (Pall Corporation) membranes. The blocked membranes were then incubated with the indicated antibodies, and immunoreactive bands were visualized with ECL reagent.

Luciferase assays

HaCaT cells at approximately 60–80% confluence were co-transfected with a COX-2 promoter luciferase fusion reporter construct, pNF-κB-Luc (Stratagene, USA) and pRL-TK control vector (Promega, USA) with the lipofectamine reagent (Invitrogen, USA). They were cultured in DMEM without fetal bovine serum and antibiotics for 24 h, and incubated with [6]-gingerol for 30 min prior to UVB irradiation. After 8 h, luciferase activity was measured with a luciferase reporter assay system (Promega). Normalized luciferase activity was calculated as the ratio of luciferase activity to control vector activity.

Measurement of intracellular ROS

Serum-starved cells were seeded at 3×10^5 cells/well in six-well plates, stabilized in Hanks' Balanced Salt Solution (HBSS, BioWhittaker™, USA) for at least 30 min and exposed to UVB after pre-treatment with [6]-gingerol. Three hours later, 10 μM DCFH-DA was added for 30 min and the cells were immediately observed with a laser scanning confocal microscope.

Preparation of nuclear extracts from HaCaT cells and mouse skin, and electrophoretic mobility shift assays (EMSAs)

Cells were washed once with cold PBS, scraped of the dishes and homogenized in 400 μl of buffer A (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM PMSF, protease inhibitor cocktail). The dorsal skins of mice were homogenized in 800 μl of buffer A. After incubating on ice for 15 min, nonidet P-40 (NP-40) solution was added to a final concentration of 0.6%, and the mixtures were centrifuged for 2 min at 14,000 rpm. The pelleted nuclei were resuspended in 50 μl of buffer C (20 mM HEPES (pH 7.9), 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM PMSF, protease inhibitor cocktail). After 30 min agitation at 4°C, the suspension was centrifuged for 5 min at 14,000 rpm and the supernatant containing nuclear proteins was collected and stored at −70°C (Min et al. 2005). The NF-κB oligonucleotide probe 5′-AGTTGAGGGGACTTTCCCAGGC-3′ was labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase and purified on a MicroSpin™ G-25 column (Amersham Pharmacia Biotech, Buckinghamshire, UK). Binding reactions contained 10 μg of nuclear extract with [γ-^{32}P]ATP-end labeled oligonucleotide. They were incubated for 30 min and the mixtures were resolved on native 5% polyacrylamide gels at 200 V for 2 h. Finally, the gels were dried and exposed to X-ray film.

Immunocytochemical localization of NF-κB p65

Nuclear translocation of the p65 subunit of NF-κB was examined by an immunocytochemical method. Briefly, HaCaT cells were preincubated with [6]-gingerol for 30 min before exposure to UVB. After 6 h, the treated cells were spread on slides, fixed with 3.7% paraformaldehyde, and permeabilized with 0.2% Triton X-100. After being washed in PBS, the slides were blocked with 3% bovine serum albumin for 1 h and the cells were incubated with rabbit polyclonal anti-NF-κB p65 antibody (1:100). After overnight incubation at 4°C, the cells were washed, incubated with anti-rabbit FITC-conjugated IgG (Serotec) (1:100) for 1 h, mounted with mounting medium, and observed by laser scanning confocal microscopy.

Immunohistochemical staining

The skin samples obtained from the backs of mice were fixed in 4% formalin solution overnight, embedded in paraffin by the conventional method and cut into 5 μm thick sections. For immunohistochemical staining, deparaffinized sections were treated for 15 min with 3% hydrogen peroxide in distilled water. After blocking nonspecific binding of antibody with 10% normal goat serum diluted in Tris-buffered saline with 0.1% BSA, the sections were incubated overnight at 4°C with primary goat polyclonal anti-COX-2, or rabbit anti-NF-κB p65 antibodies. Thereafter the samples were incubated with biotinylated secondary antibody and peroxidase-labeled streptavidin (DAKO, Denmark). Staining was developed with the DAB substrate chromogen system (DAKO, Denmark).

Statistical analysis

The data are presented as means ± SEM, and statistical comparisons between groups were made by unpaired 2-sided *t*-tests. All experiments were performed at least three times.

Results

Inhibition of UVB-induced ROS accumulation by [6]-gingerol in HaCaT cells

UVB irradiation induces ROS formation, which causes oxidative damage to macromolecules including nucleic acids [28]. To determine the effect of [6]-gingerol on UVB-induced ROS generation in HaCaT cells, the cells were pre-treated with [6]-gingerol (30 μM) for 30 min before UVB irradiation (100 J/m^2) and ROS were detected by confocal microscopy 3 h later. Incubation with [6]-gingerol on its own led to 97% cell survival at 10–30 μM while it caused 58–80% cell survival at concentrations exceeding 50 μM (Figure 1B). Since [6]-gingerol was not cytotoxic at 30 μM, further experiments were performed with this concentration. As shown in Figure 2, UVB caused an increase of intracellular ROS at 3 h and this effect was reduced by pre-treatment with [6]-gingerol.

ROS mediate UVB-induced COX-2 expression and NF-κB activation in HaCaT cells

ROS are able to activate diverse downstream signaling molecules and regulate the expression of genes related to UVB effects. It has been reported that ROS play an important role in the UVB-induced expression of COX-2 [29]. Since NADPH oxidase were thought to be the major source of UVB-induced ROS [17], we recently investigated the effect of Apocynin, an NADPH oxidase inhibitor, on UVB-induced COX-2 expression mediated by ROS. Apocynin, after

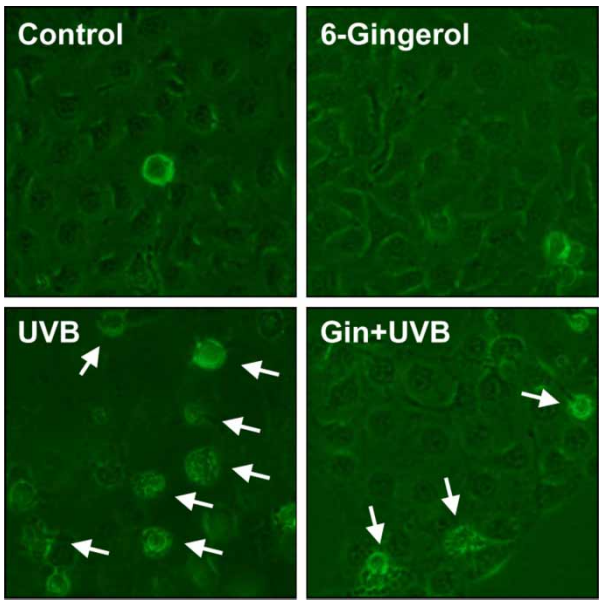

Figure 2. [6]-Gingerol reduces UVB-induced ROS in HaCaT cells. The cells were preincubated for 30 min with [6]-gingerol (30 μM). Three hours after UVB irradiation (100 J/m^2), they were labeled with 10 μM DCFH-DA for 30 min at 37°C and observed by laser scanning confocal microscopy. CTL, control untreated cells.

metabolic conversion, inhibits the assembly of NADPH oxidase [30]. As shown in Figure 3A, COX-2 expression 16 h after UVB irradiation was inhibited by Apocynin by blocking the UVB-induced generation of ROS. To determine the involvement of ROS in UVB-induced NF-κB activation in HaCaT cells, cells were pre-treated with Apocynin and *N*-acetyl cysteine (NAC), an antioxidant, before UVB irradiation. The effect of UVB on NF-κB activation was inhibited by the treatment with Apocynin and NAC (Figure 3B). These results demonstrate that ROS mediate UVB-induced COX-2 expression and NF-κB activation.

[6]-Gingerol inhibits UVB-induced COX-2 expression in HaCaT cells

It has been observed that UVB exposure increases COX-2 expression in both human skin and cultured human keratinocytes [31]. In the present experiments, UVB-induced maximal expression of COX-2 mRNA and protein at 8 and 16 h, respectively (data not shown). When HaCaT cells were pre-incubated with [6]-gingerol (30 μM) for 30 min prior to UVB irradiation the level of COX-2 mRNA 8 h after UVB irradiation decreased (Figure 4A) and the expression of COX-2 protein after 16 h was inhibited (Figure 4B). We also investigated the effect of [6]-gingerol on the transactivation of COX-2. UVB-induced COX-2 transactivation was determined by the luciferase reporter assay. Pre-treatment with [6]-gingerol inhibited UVB-induced COX-2 promoter activity (Figure 4C).

[6]-Gingerol inhibits UVB-induced activation of NF-κB and phosphorylation of IκBα

NF-κB is one of the most important transcription factors that responds directly to oxidative stress in various cell types [32] and the 5′-flanking region of the COX-2 promoter contains NF-κB binding sites [22]. To assess the effect of [6]-gingerol on UVB-induced NF-κB DNA-binding activity, we performed EMSAs with a ^{32}P end-labeled, double-stranded oligonucleotide containing the NF-κB consensus sequence. As shown in Figure 5A, UVB promoted the formation of protein DNA complexes, and [6]-gingerol inhibited their formation. To further investigate the effect of [6]-gingerol on the transcriptional activity of NF-κB, we performed NF-κB luciferase assays. Pre-treatment with [6]-gingerol attenuated UVB-induced NF-κB luciferase activity (Figure 5B). Release of NF-κB from IκBα results in nuclear translocation of NF-κB, then it binds to specific sequences in the promoter regions of target genes. We investigated the effect of [6]-gingerol on the nuclear translocation of NF-κB. UVB significantly increased nuclear translocation of the p65 subunit of NF-κB and this effect was abolished by [6]-gingerol (Figure 5C). In its inactive state, NF-κB is found in the cytoplasm bound to IκBα, which prevents it from entering the nucleus [33], and activation of NF-κB is preceded by the phosphorylation, ubiquitination, and proteolytic degradation of IκBα [34]. We examined the effect of [6]-gingerol on UVB-induced phosphorylation of IκBα by Western blot analysis using antibody against phospho-specific IκBα (Ser-32). As shown in Figure 5D, UVB irradiation led to phosphorylation of the Ser-32 residue of IκBα and [6]-gingerol inhibited this phosphorylation. Collectively, these results suggest that [6]-gingerol inhibits UVB-induced activation of NF-κB by suppressing the phosphorylation of IκBα. These results show that [6]-gingerol suppresses UVB-induced COX-2 expression by inhibiting the activation of NF-κB.

[6]-Gingerol inhibits UVB-induced COX-2 expression in mouse skin

In order to examine the effect of [6]-gingerol on UVB-induced expression of COX-2 *in vivo*, hairless mice were treated with acetone, UVB or a combination of UVB and [6]-gingerol. After UVB irradiation, skin samples from the back of each animal were obtained for analysis of COX-2 mRNA and protein. The levels of COX-2 mRNA and protein increased 8 and 16 h, respectively, after UVB irradiation. However, topical application of [6]-gingerol (30 μM) prior to UVB irradiation reduced

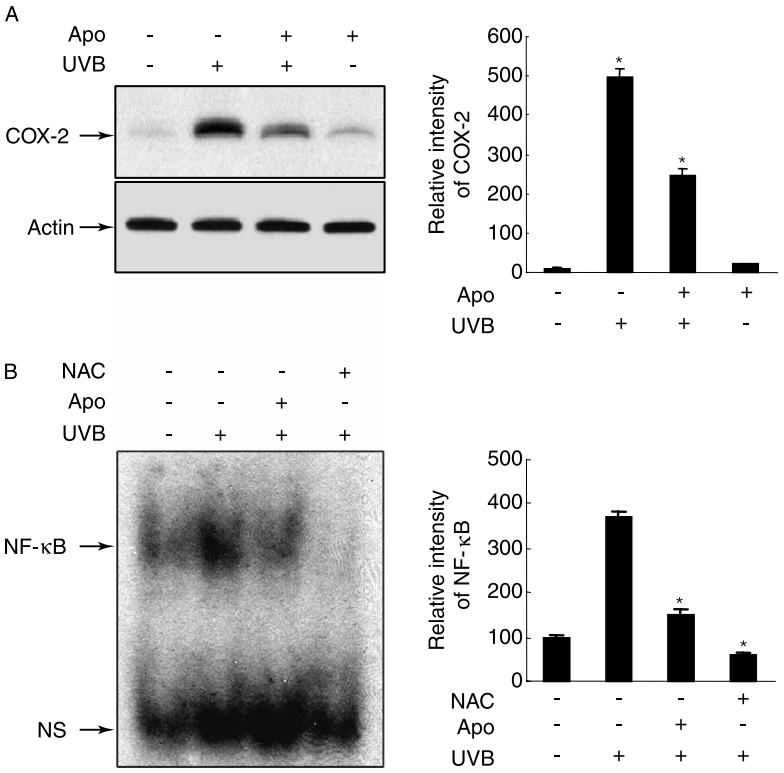

Figure 3. ROS mediate UVB-induced COX-2 expression and NF-κB activation in HaCaT cells. (A) HaCaT cells were pretreated for 1 h with Apo (Apocynin, 100 μg/ml), before exposure to 100 J/m^2 UVB. Sixteen hours after UVB irradiation, the cells were lysed and the lysates run on SDS-PAGE and transferred to a PVDF membrane. Western blots were probed with anti-COX-2 antibodies, and with anti-actin antibodies to verify equal loading of proteins. (B) HaCaT cells were preincubated for 1 or 2 h with Apocynin (100 μg/ml) or NAC (5 mM), a ROS scavenger, prior to irradiation with UVB. Six hours later, nuclear NF-κB activities were measured by EMSA as described in "Materials and Methods". A representative example of three independent experiments is shown (*$P < 0.05$).

the induced levels of COX-2 mRNA and protein (Figure 6A). Furthermore, we found by immunohistochemical analysis that UVB irradiation increased the number of COX-2 positive cells within the epidermal layer, and that this effect was reduced by pre-treatment with [6]-gingerol (Figure 6B). Therefore, [6]-gingerol inhibits UVB-induced COX-2 expression both *in vitro* and *in vivo*.

[6]-Gingerol inhibits UVB-induced activation of NF-κB in mouse skin

To confirm the effect of [6]-gingerol on UVB-induced activation of NF-κB *in vivo*, mice were topically treated with [6]-gingerol before UVB irradiation. Six hours after UVB irradiation, samples of skin were prepared for EMSAs and immunohistochemical analysis. UVB promoted the formation of protein-DNA complexes, and treatment of [6]-gingerol inhibited this effect (Figure 7A). UVB irradiation also increased the number of cells staining for NF-κB p65 and the nuclear translocation of p65, the major functionally active subunit of NF-κB, in the epidermal layer and in part of the upper dermis [35]. Topical application of [6]-gingerol reduced UVB-induced staining for NF-κB p65 (Figure 7B), confirming the inhibitory effect of [6]-gingerol on NF-κB signaling in UVB-induced COX-2 expression *in vivo* (Figure 6).

[6]-Gingerol inhibits UVB-induced caspase activation, but has no effect on UVB-induced DNA damage

The crucial importance of UVB-mediated DNA damage in UVB-induced apoptosis has been substantiated by several studies [36]. We investigated the effect of [6]-gingerol on UVB-induced DNA damage (thymine dimers) in HaCaT cells. Cells were pretreated for 30 min with [6]-gingerol (30 μM), and then exposed to 100 or 300 J/m^2 UVB irradiation. Five hours after UVB exposure, genomic DNA was extracted and analyzed by Southwestern slot–blots using an antibody against thymine dimers. UVB increased thymine dimer photoproducts in a dose-dependent manner; however, [6]-gingerol did not reduce the yield of dimers (Figure 8A). It is well known that ROS formation by UVB irradiation causes oxidative stress, which can lead to apoptotic cell death, because ROS interact with the inner mitochondrial membrane, releasing cytochrome *c* into the cytoplasm, where it activates caspases [37,38]. We, therefore, investigated the effect of [6]-gingerol on UVB-induced apoptotic cell death via both the mitochondrial

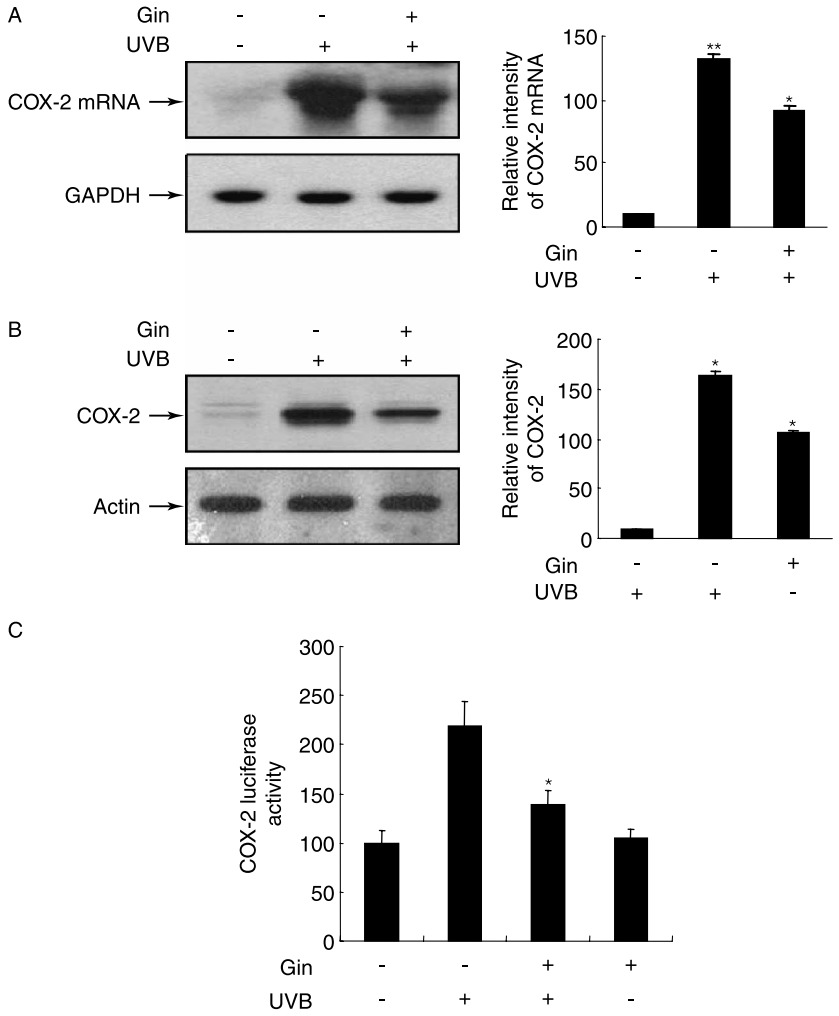

Figure 4. [6]-Gingerol inhibits UVB-induced COX-2 expression and COX-2 transactivation in HaCaT cells. HaCaT cells were pretreated for 30 min with [6]-gingerol (30 μM) before UVB irradiation (100 J/m^2). (A) Eight hours later, total RNA was extracted and COX-2 mRNA levels were determined by Northern blotting as described in "Materials and Methods" together with GAPDH mRNA as an internal standard. (B) Sixteen hours after irradiation, total cell lysates were analyzed for COX-2 expression by Western blotting, and for anti-actin to verify equal loading of proteins. The COX-2 immunoblots were normalized to those of actin followed by statistical analysis of relative image densities in comparison with control. (C) HaCaT cells were co-transfected with the luciferase COX-2 expression construct and control vector pRL-TK with lipofectamine reagent. After 24 h, they were irradiated and incubated for 8 h with or without [6]-gingerol (30 μM), while other cells were incubated with [6]-gingerol without irradiation. Luciferase activities are expressed relative to the vector-transfected cells. One representative of three independent experiments is shown ($\star P < 0.05$, $\star\star P < 0.001$).

and death receptor-dependent pathways. [6]-Gingerol reduced the activation of caspase-3, -8, -9 and Fas expression (Figure 8B). However, it had no effect on Bcl-2 and Bax levels (Figure 8B). Taken together, these results show that [6]-gingerol protects HaCaT cells from UVB-induced caspase activation.

Discussion

The skin is the largest organ of the human body, accounting for about 16% of body weight, and its barrier function is continuously challenged by environmental hazards, the most ubiquitous of which is sunlight. The UV portion of sunlight is responsible for cutaneous damage after both acute and chronic exposure, and is believed to be an important cause of skin cancers, immunosuppression and premature skin aging [39]. One approach to protecting humans from the harmful effects of UV irradiation is to use photoprotectives. In recent years, naturally occurring compounds such as phenolics have attracted considerable attention as protective agents. The phenolics, particularly polyphenols, have a variety of beneficial effects in mammals [23–25]. They can be used as components of the human diet or added to preparations for topical application. In the present study, we showed that [6]-gingerol, a pungent ingredient of ginger, suppresses the induction of ROS by UVB (Figure 2).

Recent studies have demonstrated that UVB-induced ROS cause inflammation and act as tumor promoters by a number of mechanisms including DNA damage,

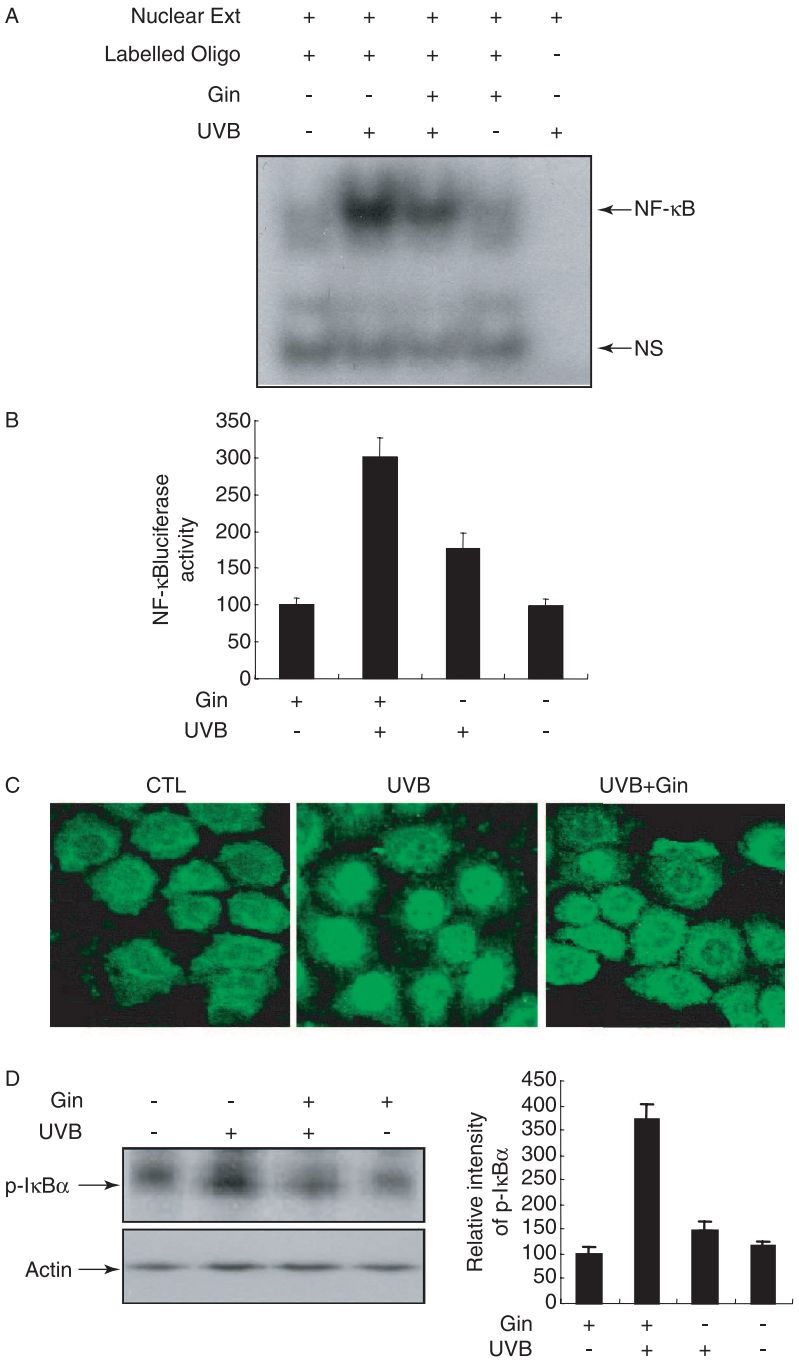

Figure 5. [6]-Gingerol inhibits UVB-induced activation of NF-κB by down-regulating phosphorylation of IκBα. (A) HaCaT cells were irradiated and incubated for 6 h with or without [6]-gingerol (30 μM), or incubated with [6]-gingerol without prior irradiation. NF-κB binding activities in nuclear extracts were measured by gel shift assays. Nuclear proteins were incubated with a [γ-^{32}P]ATP-labeled oligonucleotide containing the NF-κB consensus sequence. (B) HaCaT cells were co-transfected with pNF-κB-Luc and the pRL-TK control vector. They were then were irradiated and incubated for 8 h with or without [6]-gingerol (30 μM), or incubated with [6]-gingerol without prior irradiation. Luciferase activities are expressed relative to the vector-transfected cells. (C) Immunocytochemical analysis of p65 localization. HaCaT cells were preincubated with [6]-gingerol for 30 min before exposure to UVB (100 J/m^2). Six hours after UVB irradiation, p65 localization was analysed immunocytochemically as described in "Materials and Methods". (D) Western blot analysis of p-IκBα. Cells were UVB irradiated and incubated with or without [6]-gingerol. After 6 h, Western blots were anaysed with anti-phospho-IκBα (Ser-32) and anti-actin antibodies. A representative example of three independent experiments is shown, with control unirradiated cells set at 100% ($^\star P < 0.05$).

lipid peroxidation, and alteration of enzyme activity [40–42]. Exposure of mammalian cells to UVB stimulates signal transduction pathways, generally culminating in activation of NF-κB, a redox-regulated transcription factor that is involved in the expression of a wide variety of proinflamatory genes [43]. UVB-induced COX-2 expression is associated with erythema and infiltration of inflammatory cells

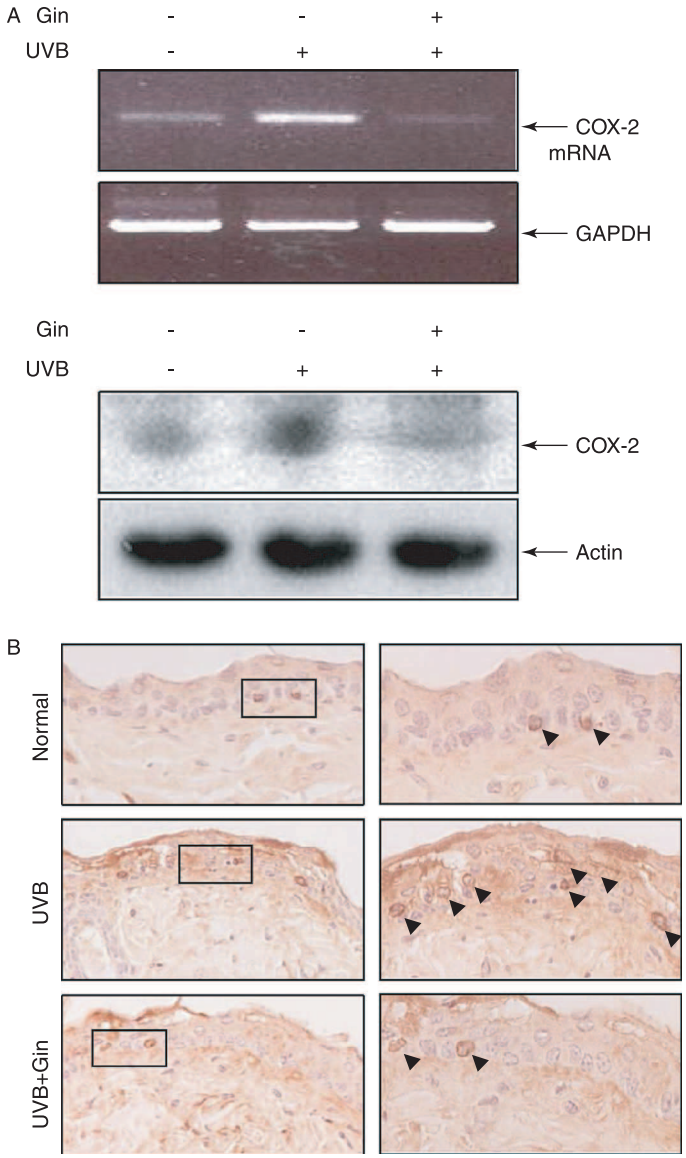

Figure 6. [6]-Gingerol inhibits UVB-induced COX-2 expression in mouse skin *in vivo*. Hairless mice were topically treated with [6]-gingerol (30 μM) dissolved in 0.2 ml acetone. After 30 min, they were exposed to UVB irradiation (5 kJ/m^2) and samples were excised from the backs of mice at the indicated times. (A) Eight hours after UVB exposure, total mRNA was isolated from mouse skins and RT-PCR performed. COX-2 mRNA and GAPDH mRNA as an internal standard were measured (upper panel). Sixteen hours after UVB irradiation, total cell lysates were analyzed for COX-2 and actin expression by immunoblotting (lower panel). (B) Mice were treated with acetone, UVB alone, or [6]-gingerol plus UVB. Sixteen hours after UVB UVB irradiation skin samples were subjected to immunohistochemical analysis using anti-COX-2 antibody as described in "Materials and Methods". Positive COX-2 staining yielded a brown-colored product (arrow).

such as mononuclear cells and neutrophils into the skin [39,44]. Therefore, COX-2 is a promising target for preventing photoinflammation and skin damage such as photoaging and tumor formation. As shown in Figure 3, ROS participate in UVB induction of COX-2 and transactivation of NF-κB. [6]-Gingerol inhibited UVB-induced expression of COX-2 mRNA and protein, and its transactivation activity in human keratinocyte HaCaT cells (Figure 4). Furthermore, we showed that it down-regulated UVB-induced DNA binding activity of NF-κB *in vitro* (Figure 5A) and *in vivo* (Figure 7A) and inhibited nuclear translocation of the p65 subunit of NF-κB by suppressing phosphorylation of IκBα (Ser-32) (Figure 5). UV-generated ROS can activate diverse downstream signaling molecules such as MAPKs and NF-κB, as well as JNK and p38 MAP kinases followed by activation of transcription factor AP-1 (activator protein 1). It has been reported that ERK and p38 MAPK are involved in mediating UVB-induced COX-2 expression in human keratinocytes [45]. UVB irradiation activated MAPK signaling pathway via phosphorylation of ERK, p38 MAPK and JNK, but, [6]-gingerol did not inhibit UVB-induced activation of MAPKs (data not shown). Thus, our results indicate that [6]-gingerol suppresses UVB-induced COX-2 expression by blocking the

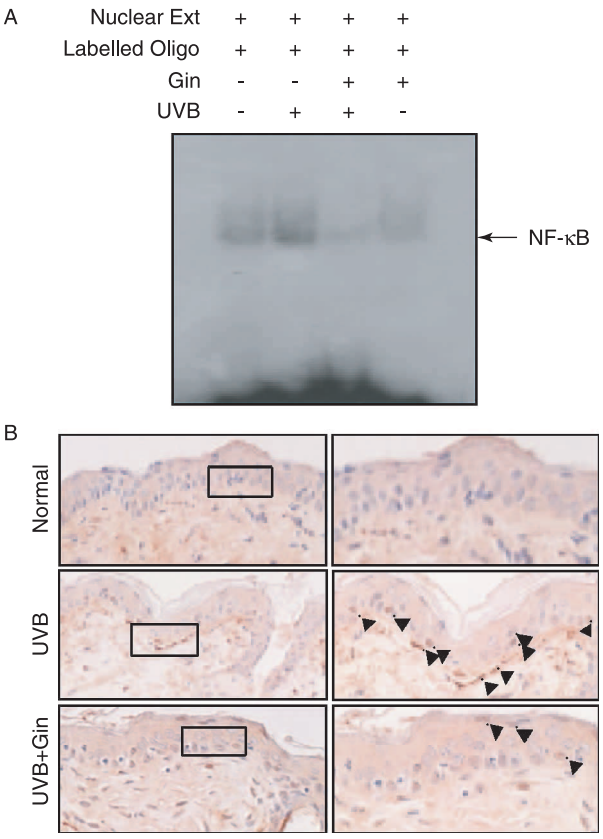

Figure 7. [6]-Gingerol inhibits UVB-induced activation of NF-κB in mouse skin *in vivo*. Hairless mice were topically treated with [6]-gingerol (30 μM) dissolved in 0.2 ml acetone. After 30 min, they were exposed to UVB irradiation (5 kJ/m^2). (A) Six hours after UVB treatment, nuclear extracts were prepared and nuclear proteins were incubated with [γ-^{32}P]ATP radiolabeled oligonucleotides containing the NF-κB consensus sequence and analysed by EMSA. (B) Six hours after UVB treatment, skin samples were subjected to immunohistochemical analysis with anti-NF-κB p65 antibody as described in "Materials and Methods". Positive NF-κB p65 staining cells yielded a brown-colored product, while red staining indicates nuclear translocation of p65 (arrow).

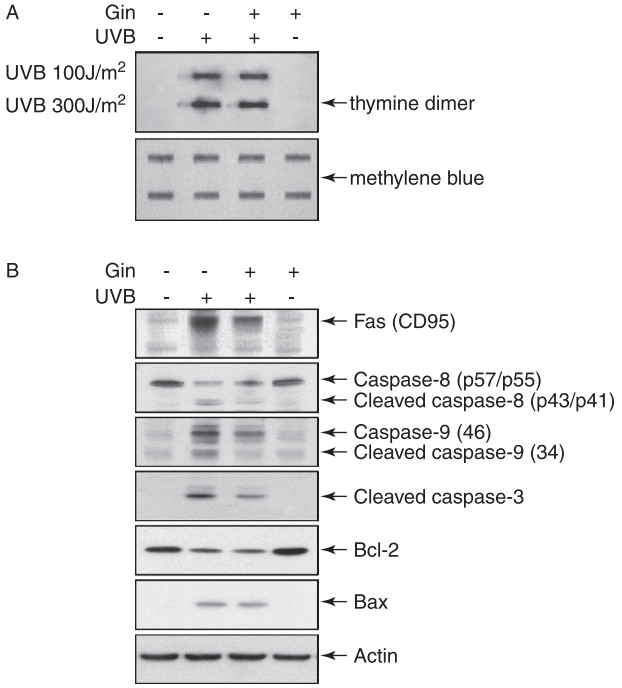

Figure 8. [6]-Gingerol down-regulates the induction of caspase-3, -8, -9 and Fas by UVB radiation but has no effect on UVB-induced DNA damage. (A) After pretreatment with [6]-gingerol, HaCaT cells were exposed to UVB irradiation (100 or 300 J/m^2). Five hours later genomic DNA was isolated and subjected to Southwestern dot–blot analysis using an antibody against thymine dimers. Methylene blue staining was used to verify use of equal amounts of DNA in the assay. (B) Cells were pretreated with [6]-gingerol (30 μM) for 30 min. Twenty-four hours after UVB irradiation, they were harvested and immunoblots analyzed with anti-cleaved caspase-3, anti-caspase- 8, -9, and anti-Fas (CD95), anti-Bax, anti-Bcl-2 antibodies, and also with anti-actin to verify equal loading of proteins.

accumulation of intracellular ROS and the activation of NF-κB in human keratinocyte HaCaT cells. These results concur with previous reports that oxidant components play an important role in COX-2 induction after UV irradiation [20,21].

[6]-Gingerol inhibits TPA-induced expression of COX-2 and its mRNA transcript in mouse skin by suppressing eukaryotic transcription factors, such as NF-κB and AP-1 [22,46,47]. We therefore investigated its effect on UVB-induced expression of COX-2 and activation of NF-κB in mouse skin *in vivo*. Topical application of [6]-gingerol (30 μM) before UVB irradiation inhibited expression of COX-2 and its mRNA (Figure 6A) as well as the increase in COX-2 positive cells within the epidermal layer (Figure 6B). We also demonstrated that [6]-gingerol inhibits UVB-induced COX-2 expression both *in vitro* and *in vivo*. *In vivo*, topical application of [6]-gingerol inhibited the increase in DNA binding activity of NF-κB, and reduced the number of NF-κB-positive cells as well as nuclear translocation of the NF-κB p65 subunit in the skin of UVB-irradiated hairless mice (Figure 7). These results demonstrate that UVB-induced ROS formation augments the expression of COX-2 and that the inhibitory effect of [6]-gingerol on UVB-induced COX-2 expression is due to inhibition of NF-κB signaling by preventing ROS accumulation in human keratinocyte HaCaT cells and mouse skin.

We also investigated the effect of [6]-gingerol on UVB-induced DNA damage. It has been reported that UVB induces three biological effects: induction of DNA damage, activation of the cell death receptor and the production of ROS [48] and that ROS subsequently induce apoptosis [49]. UV radiation activates both the extrinsic or membrane death receptor pathway and the intrinsic or mitochondrial apoptotic signaling pathways in keratinocytes [50]. UV irradiation-induced apoptosis is regulated by a number of molecular processes, including activation

of the cell death receptor CD95 (Fas/APO-1) independently of the ligand CD95L [51]. Activation of Fas by UVB irradiation results in cleavage and activation of procaspase-8, and caspase-3 is also known as a downstream effector protease that can be activated by other upstream caspases [52,53]. [6]-Gingerol significantly down-regulated the activation of caspase-3, -8, -9 and expression of Fas by UVB irradiation; however, it did not reduce UVB-induced DNA damage (thymine dimmers) (Figure 8) or apoptosis monitored by TUNEL assay (data not shown). Since we observed that [6]-gingerol did not repair or rescue cells from DNA damage, but inhibited activation of apoptotic signal, [6]-gingerol could help discriminating cells in early apoptotic event by preventing them from the cells that undergo severe damage. Skin is the multicellular organism, each cell type will show different response under UVB exposure, and accompanying phenomena will occur in different degrees. If [6]-gingerol selectively protects cells from mild damage, and then severely damaged cells will be discarded to maintain homeostasis of the skin. A hallmark of UVB exposure is the induction of apoptotic cell death in keratinocytes, and the results of which are evident within the epidermis as sunburn [54], therefore these results suggest that [6]-gingerol may have a protective effect against UVB-induced damage.

In conclusion, the development of novel preventive and therapeutic strategies is very important for protection against UVB-induced damage because human skin is constantly exposed to the UV irradiation present in sunlight and this may induce a number of pathological cellular changes. We have shown that [6]-gingerol inhibits the UVB-induced expression of COX-2 by suppressing intracellular levels of ROS and activation of NF-κB and so inhibits UVB-induced apoptotic pathway. Our findings suggest that [6]-gingerol could be an efficient agent protecting from UVB damage.

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