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Curcumin inhibits the expression of COX-2 in UVB-irradiated human keratinocytes (HaCaT) by inhibiting activation of AP-1: p38 MAP kinase and JNK as potential upstream targets

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Abbreviations: COX-2, cyclooxygenase-2; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated protein kinase; JNK, c-Jun NH2-terminal kinase; MAPKs, mitogen-activated protein kinases; ROS, reactive oxygen species; UVB, ultraviolet B

Abstract

Ultraviolet B (UVB) irradiation of skin induces an acute inflammation. Cyclooxygenase-2 (COX-2) protein plays key roles in acute inflammation in UVB-irradiated keratinocyte cell line HaCaT. Recently, curcumin has been regarded as a promising anti-inflammatory agent due to its ability to inhibit COX-2 expression. However, it remains largely unknown whether curcumin inhibits the UVB-induced COX-2 expression in HaCaT cells. This study was undertaken to clarify the effect of curcumin on the expression of COX-2 in UVB-irradiated HaCaT cells and further determined the molecular mechanisms associated with this process. In this study, we have found that the expression of COX-2 mRNA and protein were up-regulated in UVB-irradiated HaCaT cells in a dose- and time-dependent manner. Interestingly, treatment with curcumin strongly inhibited COX-2 mRNA and protein expressions in UVB-irradiated HaCaT cells. Notably, there was effective inhibition by curcumin on UVB-induced activations of p38 MAPK and JNK in HaCaT cells. The DNA binding activity of AP-1 transcription factor was also markedly decreased with curcumin treatment in UVB-irradiated HaCaT cells. These results collectively suggest that curcumin may inhibit COX-2 expression by suppressing p38 MAPK and JNK activities in UVB-irradiated HaCaT cells. We propose that curcumin may be applied as an effective and novel sunscreen drug for the protection of photoinflammation.

Keywords: COX-2; curcumin; HaCaT; MAPK; UVB

Introduction

Exposure of excessive sunlight is an important etiologic factor in the development of acute inflammation, characterized by erythema, edema, and immunosuppression, and thus consequently linked to the progression of skin cancer (Granstein and Matsui, 2004; Matsumura and Ananthaswamy, 2004). Ultraviolet B (UVB) is a well-known major risk factor for the development of acute inflammation as well as non-melanoma skin cancer in epidermis (De Fabo *et al.,* 2004; Ramos *et al.,* 2004). Accumulating data indicate that UVB exerts its detrimental effect mainly through the induction of direct DNA damage or the production of reactive oxygen species (ROS) (de Gruijl, 2002; Kulms *et al.,* 2002; Heck et al, 2003). Direct DNA damage or ROS often triggers some signaling pathways such as mitogen-activated protein kinases (MAPKs) which are known to be involved in proliferation and survival of the cells (Rhee, 1999; Torres and Forman, 2003). The MAPKs are a family of proline-directed Ser/Thr kinases composed of extracellular signal-regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK), and p38 MAPK. Recent studies have shown that activation of ERK, JNK, and p38

MAPK is tightly correlated with acute inflammation and development of skin cancer through increased expression of cyclooxygenase-2 (COX-2) (Chen *et al.,* 2001; Lin *et al.,* 2004; Mahns *et al.,* 2004).

Curcumin is a yellow pigment present in the rhizomes of turmeric (*C.longa* Linn) and related species, and has a wide array of pharmacological and biological activities including anti-inflammatory, anti-oxidant, and chemopreventive properties (Dorai and Aggarwal, 2004; Zhu *et al.,* 2004). Recently, it has been reported that curcumin-treated cells show the decreased expressions of COX-2 in various cell lines by inhibition of MAPKs signaling pathways (Chun *et al.,* 2003; Hong *et al.,* 2004; Kang *et al.,* 2004). Currently, it is unknown whether curcumin modulates the expression of COX-2 in UVB-irradiated HaCaT cells. Therefore, in this study, we investigated the effects of curcumin on the expression of COX-2 in UVB-irradiated HaCaT cells and further determined the molecular mechanisms of anti-inflammatory and anti-tumor promoting activities of curcumin.

Materials and Methods

Materials

Antibodies against phospho-ERK (p-ERK), ERK, phospho-p38 (p-p38), p38, phospho-JNK (p-JNK), and JNK were purchased from Cell Signaling (Beverly, MA). Antibodies against β-tubulin was obtained form Santa Cruz Biotechnology (Santa Cruz, CA). SB203580 and SP600125 were purchased from Biomol (Plymoth, PA, CA) and Calbiochem (La Jolla, CA), respectively. Curcumin was purchased from Sigma-Aldrich (St. Louis, MO).

Cell culture

Human keratinocyte cell line, HaCaT cell, was maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in Eagle's minimum essential medium supplemented with 10% heat inactivated fetal bovine serum, 2 mM glutamine, and 100 U/ml penicillin and 100 μg/ml streptomycin. For experiments, cells (5×10^4 cells/ml) were seeded in culture dish, and maintained in a tissue culture incubator.

UVB irradiation

As previously described (Lee *et al.,* 2003), UVB was supplied by a closely spaced array of seven Westinghouse FS-40 sunlamps, which delivered uniform irradiation at a distance of 38 cm. The energy output of UVB (290-320 nm) at 38 cm was measured with a UVB photometer (IL1350 photometer, International Light, Newburyport, MA). Cells were exposed for 0, 45, 90, and 180 sec of UVB, corresponding to doses of 0, 50, 100, and 200 mJ/cm^2. To prevent light absorption by tissue-culture medium, the culture medium was removed just prior to irradiation and replaced with a thin layer of phosphate-buffered saline (PBS) to cover the cells. Tissue culture medium was replaced in dishes immediately after the last UVB dose had been administered.

Reverse Transcription-Polymerase chain reaction (RT-PCR)

Total RNA was isolated from cells using the RNAzol™ B (Biotecx laboratories, Houston, TX) according to the manufacturer's instructions and quantitated by spectrophotometer. One microgram of total RNA was reverse transcribed using M-MLV reverse transcriptase (Promega Co., Madison, WI). The PCR reaction was carried out under the conditions recommended by the manufacturer's instructions (Takara Co., Otsu, Japan). Briefly, 50 μl of a reaction mixture including 2.5 U of Taq polymerase (Takara Co., Otsu, Japan), 5 μl of 10 × buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 1 μl of first-strand cDNA, and 25 pmol of each primer, was subjected to 28 PCR cycles (denaturation at 94°C for 1.5 min, annealing at 58°C for 1 min, and polymerization at 72°C for 1 min). The PCR products were analyzed on 1.5% agarose gel. The primer sequences and product sizes were as follows: 1) GAPDH (forward; 5'-CGTCTTCACCACCATGGAGA-3', reverse; 5'-CGGCCATCACGCCACAGTTT-3'), 300 base pair (bp); 2) COX-2 (forward; 5'-TTCAAATGAGATT-GTGGGAAAAT-3, reverse; 5'-AGATCATCTCTGCC-TGAGTATCTT-3'), 305 bp.

Western blot analysis

Whole cell extracts were prepared in the lysis buffer [10 mM Tris (pH 7.4), 5 mM EDTA, 130 mM NaCl, 1% Triton X-100, phenylmethylsulphonyl fluoride (PMSF, 10 mg/ml), aprotinin (10 mg/ml), leupeptin (10 mg/ml), 5 mM phenanthroline and 28 mM benzamidine-HCl] as described previously (Cho *et al.,* 1994). For phospho-protein detection, cells were washed with ice-cold phosphate-buffered saline containing 1 mM Na_3VO_4 and 1 mM NaF, and lysed in a buffer [20 mM Tris-Cl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM NaF, 2 mM EDTA, 200 nM aprotinin, 20 μM leupeptin, 50 μM phenanthroline, 280 μM benzamidine-HCl]. To isolate cytosolic and nuclear proteins, cells were homogenized in ice-cold hypotonic buffer (10 mM HEPES, 10 mM KCl, 3 mM $MgCl_2$, 0.5% NP-40, 2 mM PMSF, 1 mM DTT, 200 nM aprotinin) for 20 min and centrifuged at 12,000 rpm for 10 min. The supernatant was saved as a cytosolic fraction. The pellets were homogenized in ice-cold nuclear extract buffer [10 mM Tris-Cl (pH 7.5), 0.5 M NaCl, 2.5% glycerol, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT, 2 mM PMSF, 200 nM aprotinin] for 20 min and centrifuged at 12,000 rpm for 10 min. The supernatant was saved as a nuclear fraction. The protein concentration of extracts was estimated with Bradford reagent (Bio-Rad, Hercules, CA) using bovine serum albumin as the standard. Equal amounts of protein (40 μg/lane) were resolved by 10-12% sodium dodecyl sulfate-polyacrylamide gel electropho-

resis, and transferred onto a nitrocellulose membrane. The membrane was then washed with Tris-buffered saline (10 mM Tris, 150 mM NaCl) containing 0.05% Tween 20 (TBST) and blocked in TBST containing 5% non-fat dried milk. The membrane was further incubated with respective specific antibodies such as COX-2 (1:2,000), p-ERK (1:2,000), ERK (1:2,000), p-JNK (1:1,000), JNK (1:2,000), p-p38 (1:1000), p38 (1:2,000), β-actin (1:10,000), and β-tubulin (1:5,000). The membrane was continuously incubated with appropriate secondary antibodies coupled to horseradish peroxidase, and developed in the ECL Western detection reagents (Amersham Pharmacia Biotech, Piscataway, NJ).

Electrophoretic mobility shift assay (EMSA)

For extraction of nuclear extracts, 4×10^6 cells were washed with cold PBS, suspended in 400 μl of cold lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 1 μg/ml aprotinine, 30 μg/ml Leupeptin, 5 μg/ml pepstatin, 1 mM O-phenanthroline) and incubated on ice for 15 min. Then, 25 μl of 10% NP-40 were added and the tube was mixed vigorously for 10 sec, and then the homogenate was centrifuged for 30 sec at 10,000 *g* at 4°C. The nuclear pellet was resuspended in 50 μl of cold nuclear extraction buffer (20 mM HEPES pH 7.9, 0.4 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 1000 U/ml aprotinine, 30 μg/ml leupeptin, 5 μg/ml pepstatin, 1 mM O-phenanthroline) and incubated under stirring at 4°C for 15 min. It was then centrifuged for 5 min at 10,000 *g* at 4°C, and stored at -80°C until use. For EMSA assay, nuclear extracts were incubated with 15 μl binding buffer (10 mM Tris pH 8, 50 mM NaCl, 1 mM EDTA, 5% glycerol, 1 mM DTT, 2.5 mM PMSF) and 50 ng of [^{32}P]-end-labelled double-stranded activator protein-1 (AP-1) oligonucleotide (5'-CGCTTGATGAGTCAGCCGGAA-3' and 3'-GCGAACTACTCAGTCGGCCTT-5'). After incubation during 30 min at 20°C, 2 μl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 15% ficoll) were added and AP-1-DNA complexes were separated from free oligonucleotide by electrophoresis through 5% polyacrylamide gels in 0.5 × TBE at 150 V for 90 min. Gels were dried and exposed to autoradiography film.

Results

Effect of UVB on expression of COX-2

To study the effect of UVB irradiation on the expression of COX-2, HaCaT cells were exposed to UVB at doses ranging from 50 to 200 mJ/cm^2, and then cells were harvested 12 h after irradiation for Western. As shown in Figure 1A, COX-2 expressions were dramatically increased in a dose-dependent manner. Increased expression of COX-2 was clearly visualized at 6 h, following the markedly increased

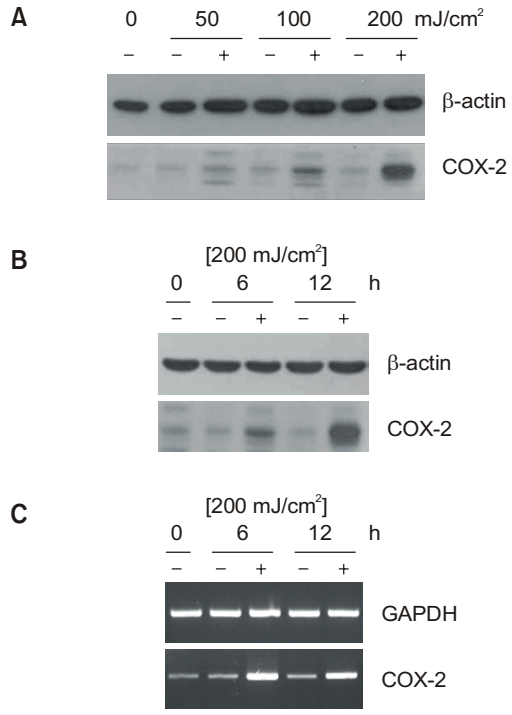

Figure 1. Up-regulation of COX-2 in UVB-irradiated HaCaT cells. The cells were cultured to 90 % confluence in DMEM supplemented with 10% fetal bovine serum at 37°C and in 5% CO_2. The cells were then serum-starved for 24 h. Before UVB irradiation, the cells were washed with PBS and were exposed to 200 mJ/cm^2 of UVB. After UVB irradiation cells were continuously cultured in serum-free media. The cells were harvested at indicated times and the cell lysates were prepared for Western blot (A, B) and RT-PCR analysis (C). Similar results were shown in two different experiments.

expression at 12 h after UVB (200 mJ/cm^2) irradiation (Figure 1B). To determine whether up-regulation of COX-2 is regulated at the level of transcription, we performed RT-PCR analysis using specific COX-2 primers. In agreement with Western, increased expression of COX-2 mRNA was clearly visualized at 6 h, following the increased expression levels were maintained at 12 h after UVB (200 mJ/cm^2) irradiation (Figure 1C). These results suggest that the up-regulation of COX-2 by UVB in HaCaT cells is largely due to increased synthesis of COX-2 mRNA.

Inhibitory effect of curcumin on UVB-induced expression of COX-2

To investigate whether curcumin inhibits the UVB-induced COX-2 expression, HaCaT cells were exposed to UVB (200 mJ/cm^2) with or without curcumin. As shown in Figure 2A, up-regulation of COX-2 by UVB was markedly decreased in a concentration-dependent manner. To investigate whether curcumin attenuates COX-2 expression by UVB at transcriptional level, we performed RT-PCR analysis using COX-2 specific primers. As shown in Figure 2B, increased

expression of COX-2 mRNA by UVB was clearly inhibited in the presence of curcumin, indicating that COX-2 expression is regulated at transcriptional level by curcumin.

Effect of curcumin on UVB-stimulated activation of MAPKs

Accumulating data suggest that UVB-irradiated HaCaT cells show an increased expression of COX-2 by MAPKs-dependent pathways, such as p38 MAPK and JNK. However it is largely unknown whether curcumin modulates the expression of COX-2 by inhibition of these MAPKs pathways in UVB-irradiated HaCaT cells. We examined the effect of UVB on activations of p38 MAPK and JNK in HaCaT cells. As shown in Figure 3A, irradiation of UVB (200 mJ/cm^2) resulted in phosphorylation (activation) of both kinases. Activation of both kinases became apparent at 5 min following irradiation of UVB. Stripping and reprobing the same membrane with antibodies against p38 MAPK and JNK revealed no change in total protein levels of each kinase, indicating that UVB induced activation of preexisting p38 MAPK and JNK. To confirm the p38 MAPK-dependent and JNK-dependent COX-2 up-regulation, HaCaT cells were pretreated with SB203580 (20 μM) and SP600125 (20 μM) and then the cells were irradiated by UVB. p38 inhibitor SB203580 and JNK inhibitor SP600125 specifically inhibited the activation of p38 and JNK, respectively (Figure 3B). We next investigated whether curcumin (10 μM) inhibits the activation of p38 MAPK and JNK in UVB-irradiated HaCaT cells. As shown in Figure 3C, phosphorylation of p38 MAPK and JNK by UVB was markedly decreased in curcumin-treated HaCaT cells. Furthermore UVB-induced COX-2 expressions were dramatically attenuated by pretreatment of SB203580, SP600125, or curcumin (Figure 3D), indicating that curcumin attenuates the UVB-induced COX-2 expression through inhibition of p38 MAPK and JNK activation. However, curcumin

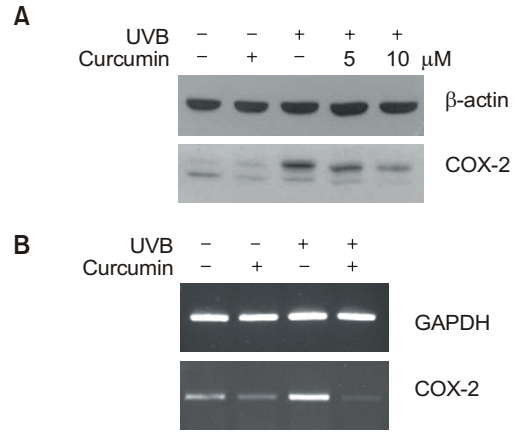

Figure 2. Inhibition of UVB-induced COX-2 expression by curcumin. The cells were irradiated by UVB (200 mJ/cm^2) with or without curcumin. The expressions of COX-2 protein and mRNA were examined by Western blot (A) or RT-PCR analysis (B), respectively. Similar results were shown in two experiments.

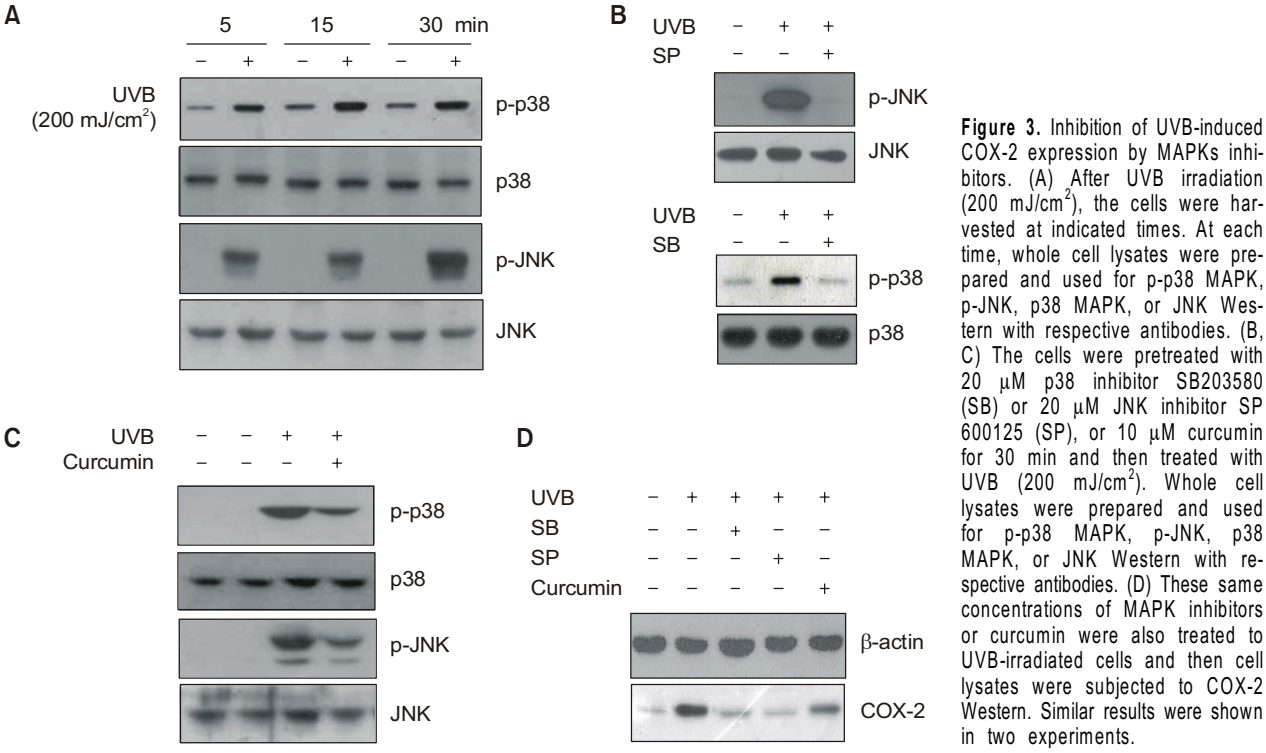

Figure 3. Inhibition of UVB-induced COX-2 expression by MAPKs inhibitors. (A) After UVB irradiation (200 mJ/cm^2), the cells were harvested at indicated times. At each time, whole cell lysates were prepared and used for p-p38 MAPK, p-JNK, p38 MAPK, or JNK Western with respective antibodies. (B, C) The cells were pretreated with 20 μM p38 inhibitor SB203580 (SB) or 20 μM JNK inhibitor SP 600125 (SP), or 10 μM curcumin for 30 min and then treated with UVB (200 mJ/cm^2). Whole cell lysates were prepared and used for p-p38 MAPK, p-JNK, p38 MAPK, or JNK Western with respective antibodies. (D) These same concentrations of MAPK inhibitors or curcumin were also treated to UVB-irradiated cells and then cell lysates were subjected to COX-2 Western. Similar results were shown in two experiments.

did not completely inhibit the activation of p38 MAPK or JNK after UVB irradiation, curcumin showed a partial inhibitory effect on the expression of COX-2 compared to SB203580 or SP600125.

Effect of curcumin on UVB-induced activation of AP-1

AP-1, a down-stream molecule of p38 and JNK, plays a key role as a transcription factor involved in UVB-induced COX-2 expression. To investigate whether curcumin decreases the AP-1 binding activity in UVB-irradiated HaCaT cells, we performed an EMSA using 21 bp consensus oligonucleotides. Exposure of HaCaT cells to UVB (200 mJ/cm^2) led to an activation of AP-1 dramatically as compared to control (Figure 4A). However, in the presence of curcumin, UVB-induced AP-1 activation was dramatically inhibited, evidenced by decreased AP-1 binding activity (Figure 4B). These results suggest that UVB-induced p38 and JNK activations are inhibited by curcumin treatment, and subsequently resulting in decreased activation of AP-1 in nucleus, thereby leading the down-regulation of COX-2 expression in HaCaT cells.

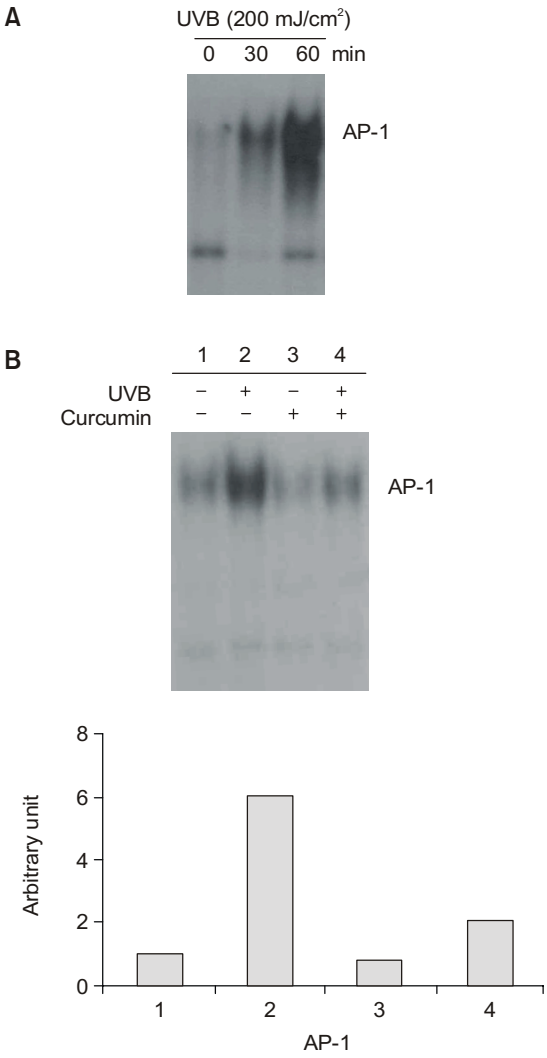

Figure 4. Suppression of UVB-induced AP-1 activation by curcumin. (A) After UVB irradiation (200 mJ/cm^2), nuclear extracts were prepared and AP-1 DNA binding activity was determined by EMSA. (B) The cells were also pretreated with curcumin (10 μM) and were exposed to UVB (200 mJ/cm^2). Nuclear extracts were prepared and AP-1 DNA binding activity was determined by EMSA.

Discussion

COX-2 plays important roles in the development of carcinogenesis as well as inflammation in UVB-irradiated skin (Grandjean-Laquerriere A *et al.,* 2002; Surh YJ, 2003; Wilgus *et al.,* 2003; Cui *et al.,* 2004; Nijsten *et al.,* 2004). Curcumin is known to exert its anti-inflammatory action in various cell lines through inhibition of COX-2 expression (Chun *et al.,* 2003; Kang *et al.,* 2004). Thus, it is important to develop an efficient strategy using curcumin to down-regulate the expression of COX-2 in UVB-irradiated skin (Wolf *et al.,* 2003).

Accumulating evidences suggest that curcumin inhibits the expression of COX-2 in several experimental models (Zhang *et al.,* 1999; Chun *et al.,* 2003; Surh, 2003; Kang *et al.,* 2004). Consistent with these, through the present study, we have demonstrated that curcumin also effectively inhibits the expressions of COX-2 mRNA and protein induced by UVB irradiation in a human keratinocyte cell line HaCaT, which seems to be, to our knowledge, the first report. Accordingly, the expression of COX-2 has been shown to be affected by various intracellular signaling proteins such as p38 MAPK and JNK (Goel *et al.,* 2001; Surh *et al.,* 2001; Liu *et al.,* 2003; Lin *et al.,* 2004; Kang *et al.,* 2004). Interestingly, it has been recently shown that UVB induces the expression of COX-2 by activation of p38 MAPK in HaCaT cells (Chen *et al.,* 2001) or by activations of p38 MAPK and JNK in artificial epidermis (Mahns *et al.,* 2004). In this study, we have observed that p38 MAPK and JNK were dramatically activated in UVB-irradiated HaCaT cells. Of importance, we have found in this study that pretreatment with curcumin effectively suppresses UVB-induced activation of p38 MAPK and JNK as well as expression of COX-2, thus suggesting that curcumin may exert its inhibitory effect on UVB-induced COX-2 expression by inhibiting p38 MAPK and JNK activities. In most cases, p38 MAPK and JNK activations in response to various extracellular stimuli are linked to activation of AP-1 (Minden *et al.,* 1994; Davis, 1995; Minden and Karin, 1997). AP-1 is known to regulate the expression of COX-2 in many systems (Dori and Aggarwal, 2004; Lin *et al.,* 2004; Kang *et al.,* 2004). In this study, we have observed that UVB is able to increase AP-1 DNA binding activity in HaCaT cells that is attenuated by treatment with curcumin. These results may suggest that curcumin may suppress AP-1 activity pro-

bably by inhibition of p38 MAPK and JNK activation in HaCaT cells. Taken together, our data suggest that suppressions of p38 MAPK, JNK, and AP-1 activity are an important molecular mechanism underlying curcumin-mediated down-regulation of COX-2 in UVB-irradiated HaCaT cells. It should be noted that curcumin can inhibit phorbol ester-induced expression of COX-2 in mouse skin through suppression of ERK activity (Chun *et al.,* 2003). However, in this study, we have observed that though UVB is able to activate ERK in HaCaT cells, curcumin does not prevent the activation of ERK in response to UVB irradiation in these cells (data not shown). Thus, molecular mechanisms underlying suppression of COX-2 expression by curcumin may be dependent on the contexts of cells or kinds of stimuli treated to cells.

In conclusion, findings of the present study demonstrate for the first time that curcumin inhibits the expression of COX-2 mRNA and protein in UVB-irradiated HaCaT cells, and indicate that the inhibitory effect of curcumin on UVB-induced COX-2 expression is likely to be, at least in part, associated with suppression of JNK, p38 MAPK, and AP-1, thereby suggesting that curcumin may be used as a promising sunscreen substance.

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