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ARTICLE

Tangeretin Reduces Ultraviolet B (UVB)-Induced Cyclooxygenase-2 Expression in Mouse Epidermal Cells by Blocking Mitogen-Activated Protein Kinase (MAPK) Activation and Reactive Oxygen Species (ROS) Generation

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The present study examined the effects of tangeretin, a polymethoxylated flavonone present in citrus fruits, on ultraviolet B (UVB)-induced cyclooxygenase-2 (COX-2) expression in JB6 P+ mouse skin epidermal cells. Tangeretin suppressed UVB-induced COX-2 expression and transactivation of nuclear factor-κB and activator protein-1 in JB6 P+ cells. Moreover, tangeretin blocked UVB-induced phosphorylation of Akt and mitogen-activated protein kinases (MAPKs), including extracellular signal-regulated protein kinase, c-Jun N-terminal kinase, and p38, and attenuated the phosphorylation of MAPK kinases 1/2, 3/6, and 4. Tangeretin also limited the endogenous generation of reactive oxygen species (ROS), thereby protecting the cells against oxidative stress. However, tangeretin did not scavenge the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and influence the nicotinamide adenine dinucleotide phosphate oxidase activity. These results suggest that the anti-inflammatory effects of tangeretin stem from its modulation of cell signaling and suppression of intracellular ROS generation. Tangeretin may have a potent chemopreventive effect in skin cancer.

KEYWORDS: Tangeretin; ultraviolet B radiation; cycolooxygenase-2; mitogen-activated protein kinase; reactive oxygen species

INTRODUCTION

Skin cancer is the most common form of cancer in humans and has the highest incidence among all cancers in the U.S. Previous studies have reported that exposure to ultraviolet (UV) radiation increases the risk for the development of skin cancer (*1*). UVB radiation is the primary cause of sunburn and many skin disorders, including cancer. Numerous studies have demonstrated the carcinogenic effects of UVB radiation, which is reported to be involved in the initiation, promotion, and progression of skin cancer (*2*, *3*).

Cyclooxygenase-2 (COX-2) is the rate-limiting enzyme in the conversion of arachidonic acid to prostaglandins. COX-2 expression is induced by inflammatory cues in various tissues, including the epidermis (*4*, *5*). Chronic inflammation is an important tumor-promoting factor, and lots of studies suggest that aberrant COX-2 expression is a key factor in inflammation and carcinogenesis (*6*−*10*). Previous studies have shown that chronic exposure to UVB radiation increases COX-2 expression via various cell-signaling pathways, resulting in the aggravation of skin cancer (*11*, *12*).

COX-2 may be upregulated through the activation of various signaling pathways. Excessive and chronic COX-2 activation, which has been shown to be mediated by mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI3K)/Akt, as well as transcription factors, such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB), results in inflammation and promotes tumorigenesis (*13*). UVB activates extracellular signal-regulated protein kinase (ERK), c-Jun N-terminal kinase (JNK), and p38 MAPK signaling pathways, which transactivate various transcription factors, such as AP-1 and NF-κB (*14*, *15*). The COX-2 promoter contains binding sites for AP-1 and NF-κB (*13*, *14*, *16*), and it has also been reported that activation of Akt contributes to COX-2 expression (*17*). The inhibition of these signaling pathways might be one of the effective strategies for the prevention of skin cancer.

Numerous factors induce oxidative stress through the activation of specific signaling pathways (*18*). Reactive oxygen species (ROS) are small molecules that react with cellular components and produce structural and functional damage in cells and tissues. They have been further identified as mediators of carcinogenesis (*19*). It is also elucidated that the cutaneous ROS production can be induced by UVB radiation (*20*, *21*). The exposure to UVB leads to cellular damage and subsequently modulates the activation of signaling pathways, including those involving MAPKs (*22*, *23*). It has been reported that increased ROS levels enhance UVB-induced skin carcinogenesis (*23*) and that ROS stimulate COX-2 (*24*, *25*). Thus, reducing intracellular ROS levels or

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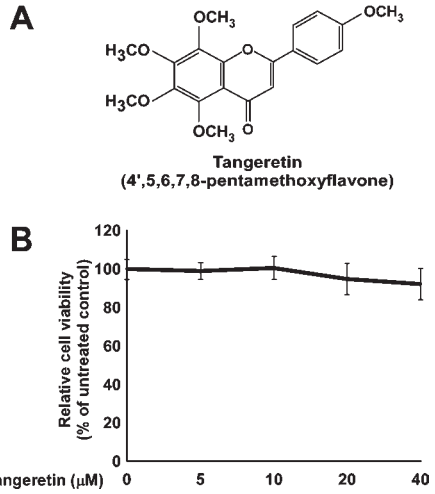

Figure 1. Tangeretin structure and effects on cell viability. (A) Chemical structure of tangeretin. (B) Effect of tangeretin on cell viability. JB6 P+ cells were treated with the indicated concentrations of tangeretin for 24 h. Cell viability was determined by the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, as described in the Materials and Methods. Data represent the mean ± standard deviation (SD) of three independent experiments.

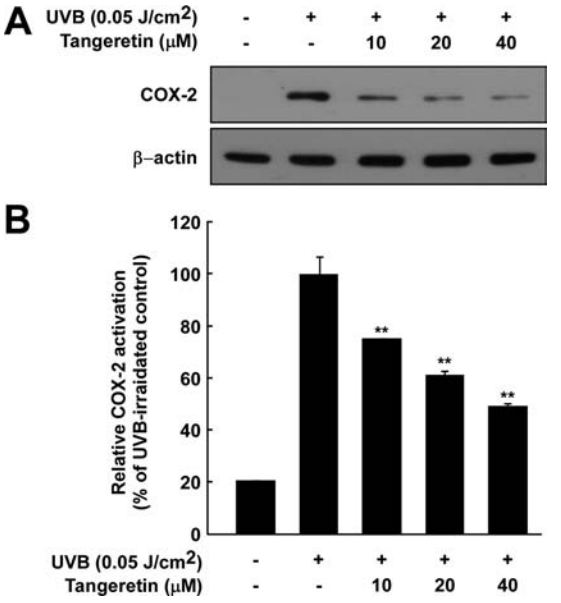

Figure 2. Effect of tangeretin on UVB-induced COX-2 expression and *cox-2* promoter activity. (A) Tangeretin inhibited UVB-induced COX-2 expression in JB6 P+ cells. Cells were pretreated with tangeretin (10, 20, or 40 μM) for 1 h, exposed to 0.05 J/cm^2 UVB, and then harvested 6 h later. COX-2 and β-actin protein levels were measured by western blot analysis using specific antibodies as described in the Materials and Methods. (B) Tangeretin inhibited UVB-induced *cox-2* promoter activity. JB6 P+ cells stably transfected with a COX-2 promoter-luciferase reporter plasmid were cultured, serum-starved in 0.1% FBS−MEM, pretreated with tangeretin (10, 20, or 40 μM) for 1 h, exposed to UVB radiation (0.05 J/cm^2), and harvested 6 h later. Relative luciferase activity was determined as described in the Materials and Methods. Data represent the mean ± SD. (*) $p < 0.05$ and (**) $p < 0.01$ versus the UVB-treated control.

blocking ROS generation may represent an effective strategy for preventing inflammatory diseases.

Flavonoids are natural compounds found in fruits and vegetables. They are widely known to help prevent the development of various diseases, including cancer. Tangeretin (4′,5,6,7,8-pentamethoxyflavone; **Figure 1A**) is a polymethoxylated flavonone present in the peel of citrus fruits. Previous studies have reported that tangeretin has a wide range of biological activities and produces antiproliferative and anticarcinogenic effects (*26*, *27*). However, its role in skin carcinogenesis remains unclear. Here, a series of experiments are conducted to examine the effects of tangeretin on UVB-induced COX-2 expression in JB6 P+ mouse epidermal cells. In the result, tangeretin inhibited UVB-induced COX-2 expression by attenuating intracellular ROS generation and subsequently suppressed MAP kinase and Akt signaling, without affecting NADPH oxidase activity.

MATERIALS AND METHODS

Materials. Tangeretin was purchased from Chromadex (Irvine, CA). Eagle's minimal essential medium (MEM) was obtained from Cellgro (Manassas, VA). Fetal bovine serum (FBS), anti-β-actin antibody, and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, MO). Anti-COX-2 antibody was obtained from Cayman (Ann Arbor, MI). Antibodies against phosphorylated JNK (Thr183/Tyr185), MAPK kinase 3/6 (MKK3/6), MAPK kinase 4 (MKK4), MAPK/extracellular signal-regulated kinase 1/2 (MEK1/2) (Ser217/221), Akt (Ser473), total Akt, MEK1/2, and MKK3 were purchased from Cell Signaling Biotechnology (Beverly, MA). Antibodies against phosphorylated p38 MAPK (D-8), ERK1/2 (E-4), total ERK1 (K-23), JNK1 (FL), MEK-4 (C-20), and p38 MAPK (A-12) were from Santa Cruz (Santa Cruz, CA). The MTT powder was purchased from USB Co. (Cleveland, OH). Penicillin/streptomycin was purchased from Gibco BRL (Grand Island, NY). Protein assay kits were obtained from Bio-Rad Laboratories (Hercules, CA).

Cell Culture. JB6 P+ mouse epidermal cells were cultured in monolayers at 37 °C in a 5% CO_2 atmosphere in MEM supplemented with 5% FBS and penicillin/streptomycin. Cells were stably transfected with luciferase reporter plasmids carrying COX-2, AP-1, or NF-κB reporter sequences. Cells were maintained in 5% FBS−MEM containing 200 μg/mL G418.

UVB Irradiation. A UVB irradiation system with a spectral peak at 312 nm (Bio-Link crosslinker, Vilber Lourmat, Cedex, France) was used to stimulate JB6 P+ cells growing in serum-free medium. Cells were exposed to 0.05 J/cm^2 of UVB and then cultured for various periods of time.

Cell Viability. Cell viability was measured by the MTT assay, which measures the mitochondrial reduction of MTT to formazan, according to the protocol of the manufacturer (Roche Diagnostics, Mannheim, Germany). JB6 P+ mouse epidermal cells in 96-well plates were cultured in 5% FBS−MEM containing penicillin/streptomycin at 37 °C in a 5% CO_2 atmosphere and were then serum-starved for 24 h. The cells were cultured with various concentrations of tangeretin for 24 h. After incubation, the cells were mixed with MTT solution (final concentration of 1 mg/mL) and cultured for a further 2 h. The dark blue formazan crystals formed in intact cells were dissolved in dimethyl sulfoxide (DMSO). Absorbance at 570 nm was measured with a microplate reader (Molecular Devices, CA) and was compared between tangeretin-treated and untreated cells.

Western Blot Analysis. Cells were cultured for 48 h and incubated in 5% FBS−MEM for an additional 24 h. They were then treated with or without tangeretin (10, 20, or 40 μM) for 1 h before being exposed to 0.05 J/cm^2 UVB radiation and were harvested at various time points thereafter. The cells were treated with lysis buffer [10 mM Tris (pH 7.5), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 1 mM dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF), 10% glycerol, and a protease inhibitor cocktail tablet] on ice for 30 min, scraped, and then centrifuged at 18620*g* for 10 min. Supernatant protein concentrations were measured using a dye-binding protein assay kit according to the guidelines of the manufacturer (Bio-Rad Laboratories, Hercules, CA). Protein samples (40 μg) were separated by 10% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and electrophoretically transferred to PVDF membranes (Millipore Corp., Bedford, MA). The membranes were blocked in 5% skim milk for 2 h and incubated overnight at 4 °C with primary antibody. After subsequent incubation with secondary antibody, the antibody-bound protein was detected using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ).

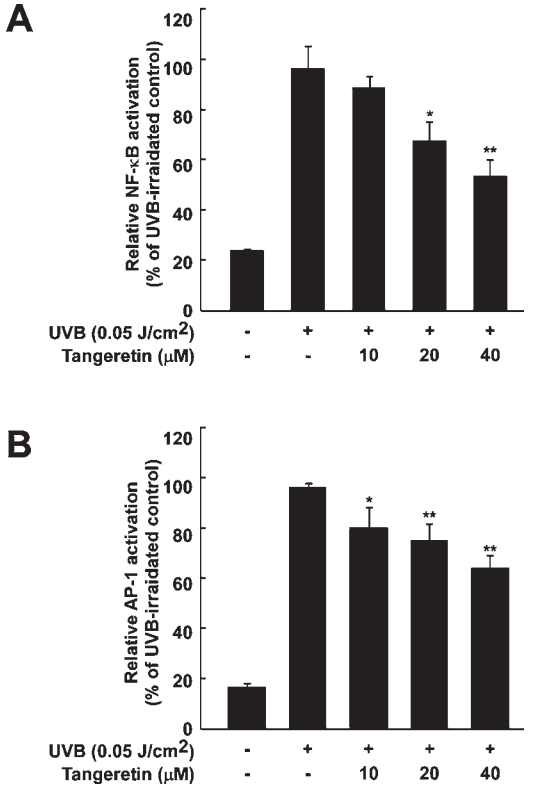

Figure 3. Effect of tangeretin on UVB-induced transactivation of NF-κB and AP-1. Tangeretin inhibited UVB-induced transactivation of (A) NF-κB and (B) AP-1. JB6 P+ cells stably transfected with NF-κB or AP-1 promoter-luciferase reporter plasmid were cultured, serum-starved in 0.1% FBS−MEM, and pretreated with tangeretin (10, 20, or 40 μM) for 1 h. The cells were exposed to UVB irradiation (0.05 J/cm^2) and harvested 6 h later. Data represent the mean ± SD. (*) $p < 0.05$ and (**) $p < 0.01$ versus the UVB-treated control.

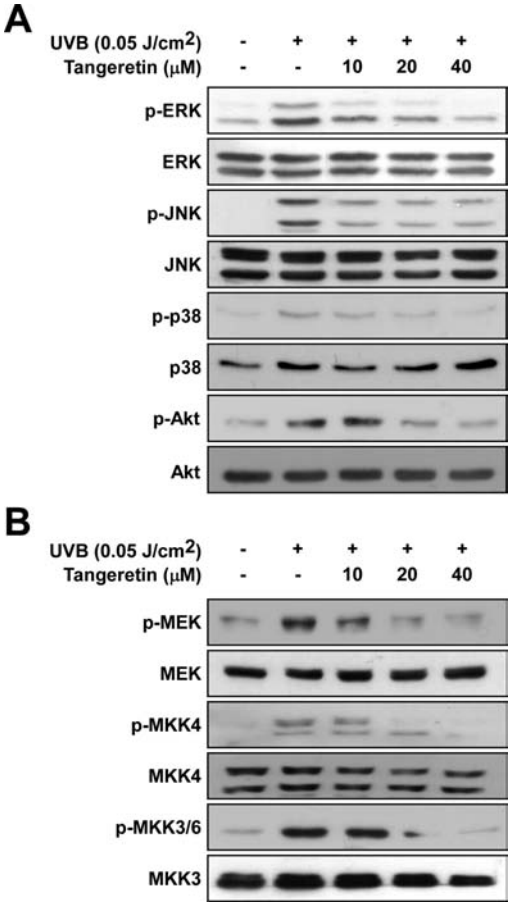

Figure 4. Effect of tangeretin on UVB-induced phosphorylation of MAP kinase and Akt pathways in JB6 P+ cells. (A) Tangeretin inhibited UVB-induced phosphorylation of ERK, p38 MAPK, JNK, and Akt. (B) Tangeretin inhibited UVB-induced phosphorylation of MEK1/2, MKK4, and MKK3/6. Cells were serum-starved in 0.1% FBS−MEM and then pretreated with tangeretin (10, 20, or 40 μM) for 1 h before being exposed to 0.05 J/cm^2 UVB radiation. The cells were harvested (B) 15 min or (A) 30 min later. Western blotting was performed using the indicated primary antibodies. Data are representative of three independent experiments with similar results.

Luciferase Assay for COX-2, NF-κB, and AP-1 Transcriptional Activity. Confluent monolayers of JB6 P+ cells stably transfected with a plasmid carrying the COX-2, AP-1, or NF-κB promoter and a luciferase reporter gene were harvested, 8×10^3 viable cells suspended in 100 μL of 5% FBS−MEM, and transferred to a 96-well plate (100 μL/well). Plates were incubated at 37 °C in a humidified 5% CO_2 atmosphere. At 80−90% confluence, the cells were incubated in 0.1% FBS−MEM for 24 h, treated with or without tangeretin (10, 20, or 40 μM) for 1 h, exposed to UVB (0.05 J/cm^2), and harvested 6 h later. Cells were disrupted in 100 μL of lysis buffer [0.1 M potassium phosphate (pH 7.8), 1% Triton X-100, 1 mM DTT, and 2 mM EDTA], and *cox-2*, *AP-1*, or *NF-κB* promoter activity was assessed by measuring luciferase activity using a Veritas microplate luminometer (Turner Biosystems, Sunnyvale, CA).

Measurement of Intracellular ROS Accumulation. Intracellular generation of ROS was measured by the DCFH-DA assay. After its deacetylation in cells, DCFH-DA is converted by intracellular radicals to a fluorescent product, DCF, which is retained intracellularly. JB6 P+ cells were cultured in 6 cm and 96-well plates for 48 h and then serum-starved for 24 h. The cells were pretreated with or without tangeretin (10, 20, or 40 μM) for 1 h, exposed to 0.05 J/cm^2 UVB, washed with PBS, and incubated in PBS containing DCFH-DA (20 μg/mL) for 20 min. Fluorescence from the cells in 60 cm or 96-well plates was measured using a real-time cell observer (Carl-Zeiss, Germany) or a fluorescence microplate reader (Molecular Devices, CA) (excitation, 485 nm; emission, 535 nm), respectively.

1,1-Diphenyl-2-picrylhydrazyl (DPPH) Scavenging Activity Assay. A solution of 0.1 mM DPPH in 80% methanol was prepared. The solution was stirred for 20 min, and its concentration was adjusted with 80% methanol to give an absorbance of 1.00 ± 0.020 at 517 nm. To 2.9 mL of the methanolic DPPH solution was added 0.1 mL of tangeretin or vitamin C solution, and the mixture was shaken vigorously for 30 min in the dark. Absorbance at 517 nm was measured using a microplate reader.

Measurement of NADPH Oxidase Activity. JB6 P+ cells were cultured to 80% confluence in 96-well plates, pretreated with or without tangeretin (10, 20, or 40 μM) for 1 h, and exposed to UVB (0.05 J/cm^2). Cells were then treated with Hank's balanced salt solution buffer containing the superoxide-sensitive chemiluminescent substrate lucigenin (25 μM) and nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (200 μM). After incubation for about 2 h, chemiluminescence was measured using a Veritas microplate luminometer.

Statistical Analysis. When necessary, the data were expressed as the mean ± SD, used to perform statistical analysis for a single comparison. A probability value of $p < 0.05$ was used as the criteria for statistical significance.

RESULTS

Tangeretin Suppresses UVB-Induced COX-2 Expression in JB6 P+ Cells. Many studies have shown that an abnormal increase in COX-2 expression plays an important role in UVB-induced skin cancer (*28*−*30*). To investigate the effects of tangeretin on COX-2 gene expression, cells were pretreated with 40 μM tangeretin, which is not cytotoxic to JB6 P+ cells (**Figure 1B**), and UVB-induced COX-2 expression in these cells was examined by western blot

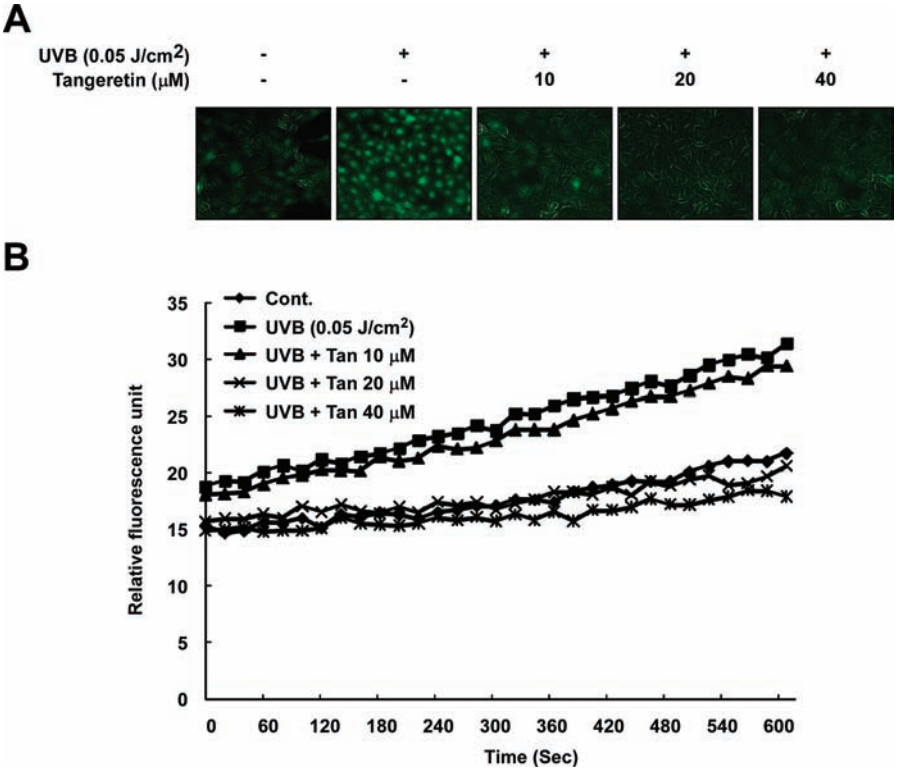

Figure 5. Effect of tangeretin on UVB-induced ROS generation. Tangeretin suppressed UVB-induced generation of intracellular ROS in JB6 P+ cells. Cells were serum-starved in 0.1% FBS−MEM, pretreated with tangeretin (10, 20, or 40 μM) for 1 h, and exposed to 0.05 J/cm^2 UVB radiation. They were then rinsed with PBS and incubated with DCFH-DA dye for 20 min. UVB-induced ROS generation was measured using (A) a real-time cell observer and (B) a fluorescence microplate reader, as described in the Materials and Methods. Data are representative of three independent experiments with similar results.

analysis. Tangeretin significantly and dose-dependently reduced UVB-induced COX-2 expression (**Figure 2A**). To confirm that tangeretin regulates COX-2 expression, the effects of tangeretin on COX-2 transcription was tested using a luciferase reporter assay. The result showed that pretreatment with tangeretin dose-dependently suppressed UVB-induced *cox-2* promoter activity (**Figure 2B**). These findings suggest that the reduction of COX-2 expression and *cox-2* promoter activity is exerted by pretreatment of tangeretin.

Tangeretin Reduces UVB-Induced *NF-κB* and *AP-1* Promoter Activity. NF-κB and AP-1 are key transcription factors in UVB-induced COX-2 expression and skin cancer (*28*, *29*). To determine whether the inhibitory effect of tangeretin on UVB-induced *cox-2* promoter activity is regulated by NF-κB and/or AP-1, *NF-κB* and *AP-1* promoter activity were measured by the luciferase reporter assay. Tangeretin dose-dependently suppressed UVB-induced transactivation of NF-κB and AP-1 (panels **A** and **B** of **Figure 3**).

Tangeretin Suppresses UVB-Induced Phosphoactivation of MAP Kinases and Akt. Previous studies have implicated the activation of Akt and MAP kinases in the induction of COX-2 expression and consequent tumor development (*13*, *31*, *32*). Therefore, the western blot was carried out to clarify the effects of tangeretin on UVB-induced phosphorylation of MAP kinases and Akt in JB6 P+ cells. Tangeretin suppressed UVB-induced phosphorylation of ERK, p38 MAPK, JNK, and Akt (**Figure 4A**). It also blocked phosphorylation of MEK, MKK4, and MKK3/6, which act upstream of MAP kinases (**Figure 4B**). These results indicate that the inhibition of MAP kinase and Akt signaling by tangeretin contributes to decreased COX-2 expression.

Tangeretin Reduces UVB-Induced Intracellular ROS Levels. ROS levels are increased by UVB radiation, resulting in oxidative cellular damage (*33*). The DCFH-DA assay was used to test whether tangeretin influences UVB-induced ROS generation. The DCF fluorescence revealed that tangeretin inhibited UVB-induced ROS generation (panels **A** and **B** of **Figure 5**), demonstrating that tangeretin reduces intracellular ROS levels in JB6 P+ cells.

Tangeretin Shows No Free Radical Scavenging Activity. We hypothesized that tangeretin might exhibit free radical scavenging activity; hence, we investigated the effect through DPPH scavenging assay, using vitamin C as a positive control. At concentrations up to 1000 μM, tangeretin did not show significant free radical scavenging activity (**Figure 6A**).

Tangeretin Does Not Inhibit UVB-Induced NADPH Oxidase Activity. A previous study demonstrated that intracellular ROS are generated by NADPH oxidase (*34*); therefore, we tested whether the inhibitory effect of tangeretin on UVB-induced ROS production results from the suppression of NADPH oxidase activity. Tangeretin did not influence UVB-induced NADPH oxidase activity in JB6 P+ cells (**Figure 6B**), suggesting that NADPH oxidase does not contribute to the attenuation of intracellular ROS generation by tangeretin.

DISCUSSION

Tangeretin is a flavonoid present in citrus fruits. Numerous studies have reported that it has various biological properties and produces anticarcinogenic, anti-inflammatory, and neuroprotective effects (*35*−*41*). However, a possible chemopreventive role of tangeretin in skin cancer has not been assessed. In the present study, it is demonstrated that tangeretin potently reduces UVB-induced COX-2 expression in JB6 P+ cells.

Chronic inflammation contributes to tumor development. COX-2, an inflammatory biomarker, provides a key link between inflammation and cancer (*7*, *42*). It has been reported that aberrant COX-2 enhanced tumor development and a lack of *cox-2* or inhibition of COX-2 suppressed tumor development (*9*, *43*, *44*). Celecoxib, a specific COX-2 inhibitor, has been shown to reduce

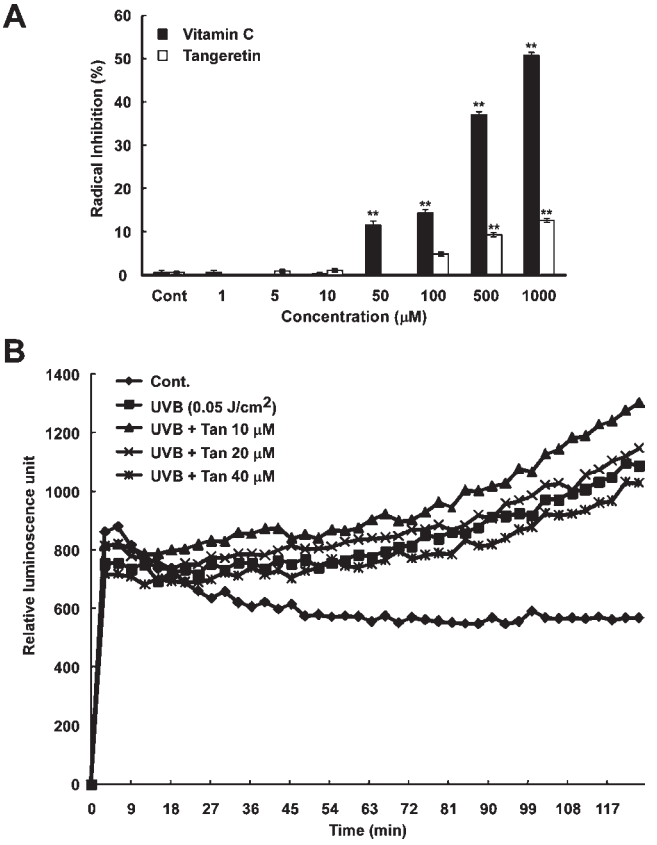

Figure 6. Effect of tangeretin on free radical scavenging and UVB-induced NADPH oxidase activity. (A) Various concentrations of tangeretin and vitamin C were used in a DPPH assay, performed as described in the Materials and Methods. Data represent the mean ± SD. (*) $p < 0.05$ and (**) $p < 0.01$ versus the untreated control. (B) Tangeretin did not suppress UVB-induced NADPH oxidase activity. Cells were cultured in serum-free MEM, pretreated with tangeretin (10, 20, or 40 μM) for 1 h, and exposed to UVB (0.05 J/cm^2). NADPH oxidase activity was measured using a microplate luminometer, as described in the Materials and Methods.

skin tumor formation in mouse models (*45*, *46*), whereas chronic upregulation of COX-2 expression and chronic inflammation can cause skin carcinogenesis (*47*). Furthermore, it has been shown that the level of COX-2 was increased in human skin cancer, and multiple studies demonstrated that UV irradiation upregulates COX-2 in cell models or *in vivo* mouse skin models (*12*, *17*, *48*−*50*). Therefore, the inhibition of COX-2 expression would be expected to suppress the development of skin cancer. In the present study, tangeretin inhibited UVB-induced COX-2 expression by suppressing *cox-2* promoter activity. This finding suggests that the inhibition of UVB-induced COX-2 expression contributes to the chemopreventive effects of tangeretin.

The transcription factors NF-κB and AP-1 regulate various biological processes, including the development of skin cancer (*51*, *52*). The activation of NF-κB and AP-1 by UVB, as a process mediated by MAP kinase signaling, triggers inflammatory responses (*53*, *54*). Celecoxib has been shown to suppress NF-κB activation induced by various carcinogens (*55*), and a second COX-2 inhibitor, SC-236, suppressed JNK activation, thereby inhibiting AP-1 activity (*56*). Previous studies have shown that both NF-κB and AP-1 regulate UVB-induced COX-2 expression in JB6 P+ cells (*57*, *58*). In the present study, tangeretin strongly inhibited UVB-induced *NF-κB* and *AP-1* promoter activity, indicating that tangeretin inhibits UVB-induced COX-2 expression by reducing the expression of NF-κB and AP-1.

It is well-known that MAP kinase and PI3K/Akt pathways are involved in UVB-induced COX-2 expression (*17*, *59*−*61*). In the present study, it has been shown that tangeretin inhibits UVB-induced phosphorylation of ERK, p38 MAPK, JNK, and Akt, as well as MEK1/2, MKK3/6, and MKK4, which act upstream of MAP kinases. The inhibition of MAP kinase and Akt signaling may be the mechanistic link between tangeretin and its attenuation of UVB-stimulated COX-2 expression.

Numerous evidence suggests that UVB radiation induces the formation of ROS, which cause cellular damage and play an important role in carcinogenesis (*23*, *33*, *62*). In this study, tangeretin strongly inhibited UVB-induced ROS generation in JB6 P+ cells but showed no ROS scavenging activity. Therefore, it is concluded that tangeretin reduces intracellular ROS levels through other mechanisms. Because tangeretin had no effect on free radical scavenging, we predicted that it may act to limit UVB-induced activation of NADPH oxidase, which converts molecular oxygen to ROS (*63*−*65*). However, this was not the case. It therefore remains unclear how tangeretin inhibits intracellular ROS production. Further studies are required to clarify the precise role of tangeretin in this process.

In summary, tangeretin limits UVB-induced COX-2 expression in JB6 P+ cells by blocking MAP kinase and Akt signaling and suppressing AP-1 and NF-κB activity and inhibits UVB-induced intracellular ROS generation. These findings suggest that tangeretin mainly suppresses the accumulation of intracellular ROS levels and subsequently reduces MAP kinase and Akt signaling, which may contribute to its protective effect in UVB-induced COX-2 expression.

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