JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

J. Agric. Food Chem. 2010, 58, 7013–7020 7013
DOI:10.1021/jf100691g

Suppression of γ-Tocotrienol on UVB Induced Inflammation in HaCaT Keratinocytes and HR-1 Hairless Mice via Inflammatory Mediators Multiple Signaling

Akira Shibata,[†] Kiyotaka Nakagawa,*[,†] Yuki Kawakami,[‡] Tsuyoshi Tsuzuki,[†] and Teruo Miyazawa[†]

[†]Food and Biodynamic Chemistry Laboratory, Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan, and [‡]Department of Nutritional Science, Faculty of Health and Welfare Science, Okayama Prefectural University, Okayama 719-1197, Japan

Tocopherol (Toc) such as α-Toc has been expected to act as photochemopreventive agent of skin, but the effect of the other vitamin E forms [tocotrienols (T3)] has not been fully understood. We evaluated the anti-inflammatory effect of T3 on UVB-induced inflammatory reaction using immortalized human keratinocytes and hairless mice. γ-T3 suppressed UVB-induced PGE_2 production while similar α-Toc doses had no effect. The anti-inflammatory actions of γ-T3 were explained by its ability to reduce UVB-induced inflammatory gene and protein expression [cyclooxgenase-2 (COX-2), interleukin (IL)-1β, IL-6, and monocyte chemotactic protein-1]. Western blot analysis revealed γ-T3 inhibited p38, extracellular signal-regulated kinase, and c-Jun N-terminal kinase/stress-activated protein kinase activation. In HR-1 hairless mice, oral T3 suppressed UVB-induced changes in skin thickness, COX-2 protein expression, and hyperplasia, but α-Toc did not. These results suggest T3 has potential use to protect against UVB-induced skin inflammation.

KEYWORDS: Cyclooxgenase-2; HaCaT; tocopherol; tocotrienol; UVB

INTRODUCTION

Ultraviolet (UV), especially UVB, is a major cause of cutaneous inflammatory disorders such as erythema and edema (*1*, *2*). These symptoms (so-called sunburn reaction) contribute to chronic diseases such as skin cancer and skin aging (*3*). Biological studies showed that UVB promotes skin inflammation by inducing production of pro-inflammatory cytokines and prostaglandins (PGs) (*4*). Therefore, agents that regulate these inflammatory molecules could provide beneficial anti-inflammatory effects. Considerable effort is being made to screen potential compounds that can reduce UVB-induced human skin inflammation. Natural compounds including epigallocatechin gallate (*5*), resveratrol (*6*), and sulforaphane (*7*, *8*) have been reported to prevent UVB-induced skin disorders *in vitro* and/or *in vivo*.

Since vitamin E (tocopherol, Toc; **Figure 1**) easily accumulates in skin tissue, its protective effect on skin damage has long been anticipated (*9*). For instance, Burke et al. showed that α-Toc reduced UVB-induced skin inflammation in hairless mice (*10*). Recently, Yamada et al. found that the other vitamin E [tocotrienol (T3); **Figure 1**] appeared to have more skin protective effect than Toc (*11*). Therefore, the effect and the mechanism of vitamin E, especially T3, on skin inflammation are interesting and require further biological evidence. Considering the study by Yoshida et al. (*12*), evaluation of interleukins (ILs), PGs, and cyclooxygenase (COX) seems to be important and necessary for further elucidation of anti-inflammatory effect and mechanism of vitamin E.

*Corresponding author. Tel: +81-22-717-8906. Fax: +81-22-717-8905. E-mail: nkgw@biochem.tohoku.ac.jp.

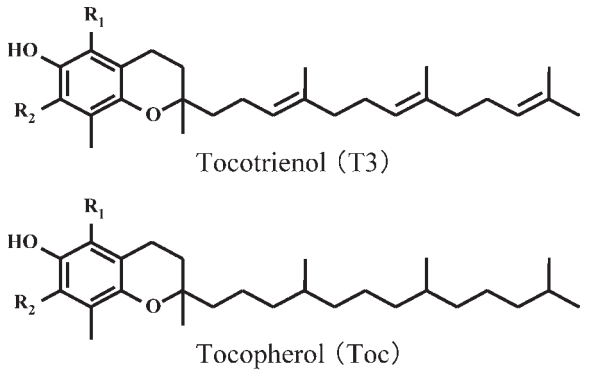
On the basis of this background, we carried out a series of investigations on the specific molecular events in UVB-induced inflammation that contribute to the anti-inflammatory effect of T3. These studies seek to clarify the different inhibitory activities of T3 and Toc, and to elucidate the effect of these two compounds on UVB-induced expression of inflammatory and signal transduction molecules that mediate keratinocyte inflammatory responses. In this study, γ-T3 was used, because our previous animal studies showed that, among T3 isomers, the γ-T3 form is the one most effectively accumulated in skin (*13*). In addition, γ-T3 is a representative T3 isomer found in natural sources such as rice bran and is easily obtained. Our findings that T3 has a novel mechanism by which it inhibits skin inflammation will provide insights into the application of T3 for anti-photoinflammatory purposes.

MATERIALS AND METHODS

Reagents, Cells, and Animals. γ-T3 was purchased from Chromadex (Santa Ana, CA). α-Toc was purchased from Sigma (St. Louis, MO) and WST-1 reagent from Dojindo Laboratories (Kumamoto, Japan). Rice bran T3 (RBT3) was kindly provided by Sanwa Yushi Co. Ltd. (Tendo, Japan). RBT3 was composed of 97.5% T3 (3.5% α-T3, 89.9% γ-T3, and 4.1% δ-T3) and 2.1% Toc (1.4% γ-Toc and 0.7% δ-Toc) (w/w). Toc-stripped corn oil was purchased from Acros Organics (Fairlawn, NJ). All other reagents were of analytical grade.

An immortalized human keratinocyte cell line (HaCaT) (*14*) was supplied by Prof. Norimichi Nakahata (Graduate School of Pharmaceutical Sciences, Tohoku University, Japan). HaCaT cells were cultured in a growth medium (Dulbecco's modified Eagle's medium, DMEM, D6046; Sigma, St. Louis, MO), supplemented with 10% fetal bovine serum (FBS) (Dainippon Pharmaceutical, Osaka, Japan), 100 kU/L penicillin, and

Published on Web 05/13/2010



	R_1	R_2
α-	CH_3	CH_3
β-	CH_3	H
γ-	H	CH_3
δ-	H	H

Figure 1. Chemical structure of T3 and Toc. T3 has an unsaturated isoprenoid tail that differs from Toc, which bears a saturated phytyl side chain. The functional groups of the different isoforms are also indicated.

streptomycin (100 mg/L) (Gibco BRL Rockville, MD) at 37 °C in 5% CO_2/95% air atmosphere in a humidified incubator.

Female HR-1 hairless mice (10 weeks of age) were obtained from Hoshino Experimental Animal Center (Yashio, Japan), and housed in cages kept at 23 °C with a 12 h light:dark cycle in pathogen-free conditions. The mice were acclimated with laboratory rodent chow (MF; Oriental Yeast, Tokyo, Japan) and water for 1 week prior to the conduct of the study.

Preparation of T3 for Cell Cultures. γ-T3 and α-Toc were dissolved in ethanol at a concentration of 20 mM. The solution was diluted with test medium (DMEM containing 0.1% FBS) to achieve the desired final concentration (0−10 μM). The final concentration of ethanol in the test medium was up to 0.1% (v/v), which did not affect cell viability. Similarly, medium containing only vehicle (0.1% ethanol) was prepared and used as a control for the study.

Sample Treatment and UVB Irradiation. HaCaT cells (1×10^4 cells/well for enzyme-linked immunosorbent assay (ELISA), $8-10 \times 10^5$ for real time reverse transcription-PCR (RT-PCR), and $4-8 \times 10^6$ for Western blot analysis) were preincubated in the growth medium (DMEM containing 10% FBS) for 24 h. Then, the medium was changed to sample free test medium (DMEM containing 0.1% FBS), and the cells were incubated for 24 h. After washing with PBS, cells in PBS were exposed to UVB (50 mJ/cm^2) delivered from an EL Lamp (8 W, UVL-28, 302 nm, 100 V (UVP); Upland, CA) that emits an energy spectrum in the UVB region with a peak at 302 nm. The emitted UVB dose was regularly measured with a UV light meter UV-340 (Lutron, Coopersburg, PA). The cells were then cultured in test medium containing γ-T3 or α-Toc for 2−24 h. UVB untreated cells were also prepared as a negative control.

PGE_2 ELISA Assay. UVB-treated and untreated cells were cultivated in test medium for 24 h, and an aliquot of the conditioned medium was taken to measure PGE_2 levels using a commercial ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The results were normalized to the number of cells per well. Cell number was evaluated by WST-1 assay (*15*). WST-1 is a tetrazolium salt that is converted into the soluble formazan salt by succinate-tetrazolium reductase in the respiratory chain of active mitochondria in proliferating viable cells. The amount of formazan produced is directly proportional to the number of viable cells. Cell number was estimated by measuring the absorbance (450/655 nm) of the medium using a microplate reader (model 550, Bio-Rad Laboratories, Inc., Hercules, CA).

Isolation of Total RNA and Analysis of mRNA Expression. UVB-treated and untreated cells were cultivated in test medium for 3 and 6 h. Total RNA was isolated from the cells using the RNeasy plus Mini kit (Qiagen, Valencia, CA) for real-time RT-PCR using DNA engine opticon 2 system (MJ Research, Waltham, MA). Then, cDNA was synthesized from the RNA using a Ready-To-Go T-Primed First-Strand Kit (GE Healthcare; Piscataway, NJ). The cDNA was subjected to PCR amplification using a SYBR Premix Ex *Taq*II (Takara Bio, Shiga, Japan) and gene-specific primers for COX-2, IL-1β, IL-6, monocyte chemotactic protein-1 (MCP-1), and β-actin (Supporting Information). PCR conditions for COX-2, IL-1β, IL-6, MCP-1, and β-actin were 95 °C for 1 min, 95 °C for 5 s, and 68 °C for 30 s over 40 cycles. A melt curve analysis was performed following each reaction to confirm the presence of only a single reaction product. In addition, representative PCR products were electrophoresed on a 2.0% agarose gel to verify that only a single band was present. The ratio between the β-actin content in control and test samples was defined as the normalization factor.

Western Blot Analysis. UVB-treated and untreated cells were cultivated in test medium for 2 and 24 h. Cellular proteins were prepared as previously described (*16*), and separated (60 μg/well) by sodium dodecyl sulfate−polyacrylaminde gel electrophoresis (SDS−PAGE, 4−20% e-PAGEL, Atto, Tokyo, Japan). The protein bands were then transferred to polyvinylidine fluoride membranes (GE Healthcare). After blocking of the nonspecific sites, the membrane was probed with primary antibodies, followed by a horseradish peroxidase-conjugated secondary antibody (Cell Signaling Technology, Beverly, MA). Antibody reactions were detected using ECL Plus Western blotting reagents (GE Healthcare). The antibodies used were anti-human COX-2 (IBL, Takasaki, Japan); antiphospho c-Jun N-terminal kinase/Stress-activated protein kinase (JNK/SAPK), anti-JNK/SAPK, antiphospho p38, anti-p38, antiphospho extracellular signal regulated kinase (ERK), anti-ERK (BD Transduction Laboratories, San Diego, CA); anti-mouse COX-2 (Cayman Chemical, Ann Arbor, MI); and anti-β-actin (Cell Signaling Technology). After the detection of protein bands, band intensities were estimated by densitometric scans.

Evaluation of Reactive Oxygen Species. The generation of intracellular reactive oxygen species (ROS) was evaluated using the fluorescent dye 2,7-dichlorodihydrofluorescein diacetate (DCDHF-DA) (Cayman Chemical) (*17*). In cells, ROS causes oxidation of DCDHF, yielding the fluorescent product 2′,7′-dichlorofluorescein. UVB-treated and untreated cells were cultivated in test medium for 3 h, whereupon the medium was changed to 0.1% FBS DMEM containing 10 μM DCDHF-DA, followed by incubation for 30 min. The cells were then washed with Hanks' balanced salt solutions, and the fluorescence intensity was determined using a GENios Plus multidetection microplate reader with enhanced fluorescence (Tecan Inc., Research Triangle Park, NC) at an excitation and emission wavelength of 485 and 535 nm, respectively.

Animal Study. The mice were divided into 4 groups: group 1, UVB-untreated/vehicle fed (Toc-stripped corn oil); group 2, UVB-irradiated/vehicle fed; group 3, UVB-irradiated/fed with 2.5 mg of α-Toc/day; and group 4, UVB-irradiated/fed with 2.57 mg of RBT3 (0.09 mg of α-T3, 2.31 mg of γ-T3, 0.1 mg of δ-T3, 0.04 mg of γ-Toc, and 0.02 mg of δ-Toc)/day. Each mouse received samples (dissolved in 50 mg of Toc-stripped corn oil) orally once a day for a total of 14 successive days. On days 9, 11, and 13, dorsal mouse skin was directly exposed to UVB irradiation from the vertical position at a dose of 200 mJ/cm^2 and a distance of 22 cm using the EL Lamp (8 W, UVL-28, 302 nm, 100 V (UVP); 12−13 min for exposure time). During the feeding period, the mice were allowed free access to water and a vitamin E-free AIN-76 diet (*18*). Skin thickness was measured daily at three midline sites using a caliper (Digimatic Micrometer 543; Mitutoyo Co., Tokyo, Japan). On day 14, 24 h after the last UVB exposure, mice were euthanized and skins were collected and analyzed as described below. These protocols were reviewed by the Committee on the Ethics of Animal Experiments of Tohoku University, and this experiment was carried out in accordance with the Animal Experiment Guidelines of Tohoku University.

Skin Analysis. Skin samples (about 50 mg) were ground under liquid nitrogen and proteins were extracted using the same procedure as described in the previous study (*16*). Extracted proteins were analyzed by Western blotting. For determination of hyperplasia, the skin was fixed in 4% formalin, and embedded in paraffin. Vertical sections (5 μm) were cut, mounted on a glass slide, and stained with hematoxylin and eosin (*19*).

Measurement of Skin T3 and Toc Concentrations. T3 and Toc in the skin were extracted as described previously (*20*). Concentrations of T3 and Toc were determined by fluorescence HPLC (FL-HPLC). Separation was performed at 35 °C using a silica column (ZORBAX Rx-SIL, 4.6 × 250 mm; Agilent, Palo Alto, CA). A mixture of hexane/1,4-dioxane/2-propanol (988:10:2) was used as the mobile phase at a flow rate of

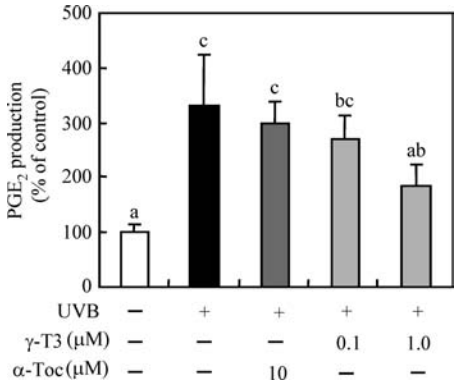


Figure 2. Effect of γ-T3 or α-Toc on UVB-induced PGE_2 secretion. UVB-treated or untreated cells were cultivated in test medium (0.1% FBS DMEM supplemented with γ-T3 or α-Toc) for 24 h, and an aliquot of the conditioned medium was used to measure the levels of PGE_2 by ELISA. Data are expressed as the percentage relative to nonirradiated control cells. Values are mean ± SD ($n = 4$). Means without a common letter differ, $P < 0.05$.

1.0 mL/min. The T3 and Toc isomers were detected and determined by an RF-10AXL FLD detector (excitation 294 nm, emission 326 nm; Shimadzu, Kyoto, Japan).

Statistical Analysis. The data are expressed as the mean ± SD. We performed statistical analysis using a 1-way ANOVA, followed by Bonferroni/Dunn test for multiple comparisons among several groups. Differences were considered significant at $P < 0.05$.

RESULTS

Effect of γ-T3 and α-Toc on UVB-Induced Inflammatory Mediator Expression. Keratinocytes characteristically produce pro-inflammatory mediators such as IL-1β, IL-6, MCP-1, and PGE_2 (*21*). In particular, PGE_2, a potent inflammatory mediator, plays a pivotal role in the acute inflammatory response of skin to UVB (*22*). We therefore first evaluated by ELISA whether or not γ-T3 or α-Toc suppresses UVB-induced PGE_2 production in HaCaT cells. Relative to nonirradiated cells, UVB-exposed HaCaT cells released higher PGE_2 levels (4-fold) (**Figure 2**). The treatment of γ-T3 (1 μM) significantly inhibited UVB-induced PGE_2 production. In contrast, α-Toc did not significantly inhibited the UVB-induced PGE_2 production.

We next measured mRNA levels of COX-2, which is involved in PGE_2 production, and also evaluated inflammatory mRNA expression for the IL-1β, IL-6, and MCP-1 in HaCaT cells. Real time RT-PCR showed that UVB irradiation significantly increased expression of these inflammatory genes (**Figure 3**). Treatment with γ-T3 (1 μM) significantly suppressed the expression of these genes. In contrast, α-Toc did not affect these genes except for MCP-1 at 6 h cultivation. Among evaluated mRNA parameters (**Figure 3**), γ-T3 showed a potent inhibitory effect on COX-2 expression. We therefore investigated COX-2 protein expression by Western blot analysis, and confirmed that 1 μM γ-T3, but not α-Toc, reduced UVB-induced COX-2 protein expression (**Figure 4A**).

Mechanism of the Anti-Inflammatory Effect of γ-T3 in HaCaT Cells. Many previous studies showed that UVB radiation induces the activation of mitogen-activated protein kinases (MAPKs), such as JNK/SAPK, p38 and ERK, which have been implicated in inflammatory signaling and lead to inflammatory mediator production (*23*). To evaluate the mechanism of γ-T3 suppression on UVB-induced inflammatory responses, we examined the effect of γ-T3 (including α-Toc) on UVB-induced MAPK phosphorylation (JNK/SAPK, p38, and ERK) by Western blot analysis using phospho-specific antibodies. As shown in **Figure 4B**, UVB radiation significantly increased phosphorylation of MAPK proteins. Treatment with γ-T3 (1 μM) inhibited all JNK/SAPK, p38, and ERK activation without altering total protein levels. On the other hand, α-Toc was not able to suppress phosphorylation of MAPK proteins.

ROS production is known to affect inflammatory cytokine secretion by stimulating signal transduction pathways (*24*). Therefore, ROS prevention may be a possible explanation for anti-inflammatory action of γ-T3. We measured ROS levels in HaCaT cells by DCDHF-DA assay, and found that γ-T3 (1 μM) decreased UVB-induced ROS generation, as α-Toc did at 10 μM (**Figure 4C**).

***In Vivo* Study of Skin Inflammation Inhibition by T3.** Finally, we evaluated the *in vivo* inhibitory effect of RBT3 (~90% γ-T3) and α-Toc on skin inflammation using the HR-1 hairless mouse model. Based on previous studies (*7*), we selected a 200 mJ/cm^2 UVB dosage to assess changes in skin inflammatory markers. Both the increase in skin thickness and sunburn reaction induced by UVB were suppressed by feeding RBT3 (**Figure 5A−C**). RBT3 also suppressed COX-2 protein expression in hairless mouse skin (**Figure 5D**). In support of this result, the severe hyperplasia detected by histological analysis of the skin of UVB-irradiated mice was attenuated by feeding RBT3 (**Figure 6**). When hyperplasia was evaluated by epidermal thickness, the thicknesses were 2−3 cell layers for UVB-non treated control mice, 10−11 cell layers for UVB irradiated mice, and 6−7 cell layers for UVB irradiated mice receiving RBT3. On the other hand, mice fed with α-Toc did not reduce UVB-induced skin thickness (**Figure 5B**) and COX-2 protein expression (**Figure 5D**). In histological analysis, the skin thickness was 8−9 cell layers for UVB irradiated mice receiving α-Toc (**Figure 6**). As shown in **Table 1**, significant incorporation of T3 and Toc in skin was observed for group 4 (UVB-irradiated/T3 2.5 mg/day) and group 3 (UVB-irradiated/α-Toc 2.5 mg/day), respectively.

DISCUSSION

In recent years, some naturally occurring compounds have gained considerable attention as skin-protective agents. This study was designed to compare the protective effects of γ-T3 and α-Toc against UVB radiation-induced damage to HaCaT cells and the skin of HR-1 hairless mice, a well-accepted animal model for photodamage studies (*5*−*7*, *11*). In this study, we demonstrated that γ-T3 suppressed UVB-induced expression of inflammatory mediators in HaCaT cells, while α-Toc had only minimal effect. This suppression appeared to be associated with the ability of γ-T3 to suppress MAPK activation and ROS production. These *in vitro* data, together with the *in vivo* results from HR-1 hairless mice, indicate that γ-T3 has a potential efficacy to ameliorate UVB-induced skin damage.

Exposure to UVB radiation induces a variety of biologic effects, including inflammation, sunburn cell formation, immunologic alterations, and photoaging (*25*, *26*). Keratinocytes, a major target of UVB radiation, play a central role in inflammatory and immunomodulatory actions. These cells are considered to be the source of UVB-induced inflammatory mediators such as cytokines, chemokines, and many others (*4*, *27*). PGE_2 is one inflammatory mediator that plays an important role in the acute inflammatory response to UVB, and is responsible for the clinical erythema seen in the sunburn response (*22*). In some experimental models, PGE_2 regulates the release of other inflammatory mediators, including IL-6 and IL-8 (*28*, *29*). For these reasons, we focused on PGE_2, and evaluated the effect of γ-T3 or α-Toc on UVB-induced PGE_2 release from HaCaT cells. In this study, we found that γ-T3 could attenuate UVB-induced PGE_2 production in HaCaT cells, while

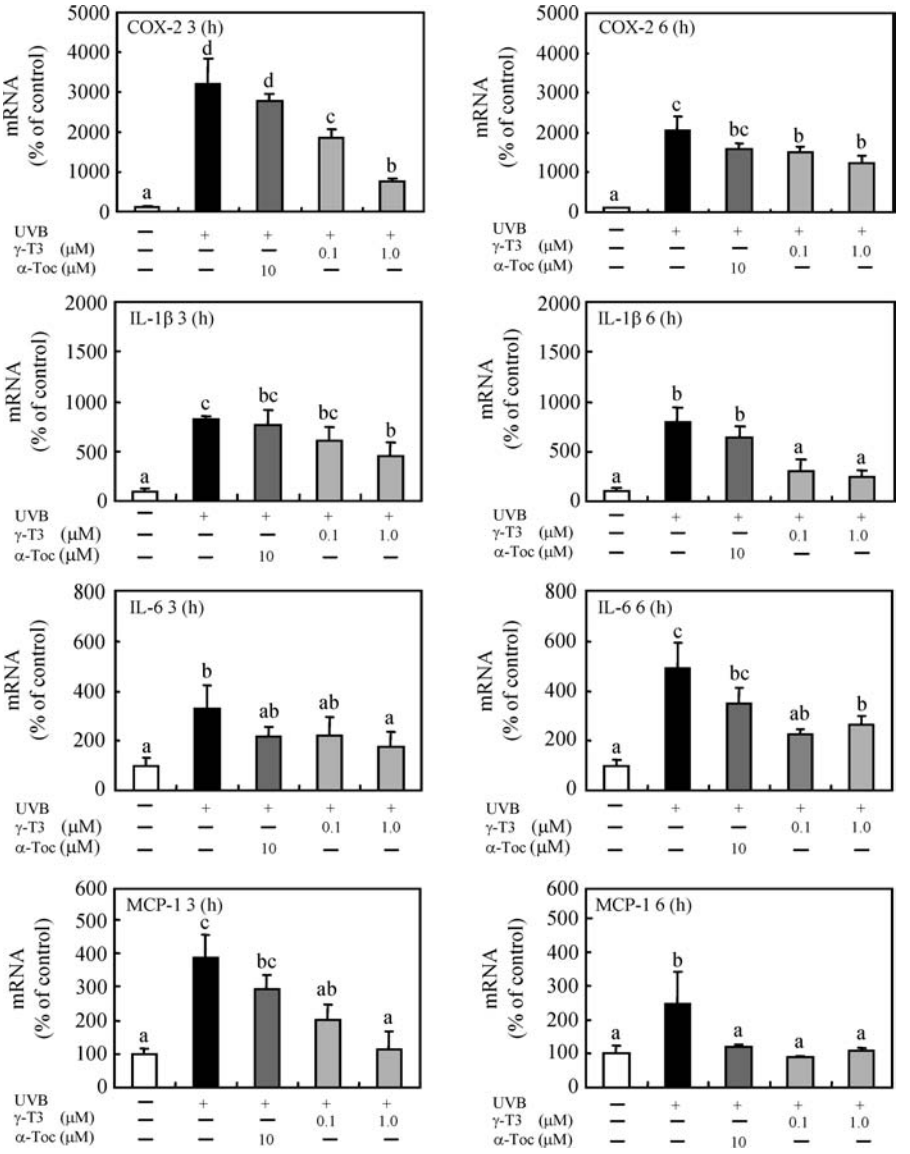


Figure 3. Effect of γ-T3 or α-Toc on UVB-induced mRNA expression of COX-2, IL-1β, IL-6, and MCP-1. UVB-treated or untreated cells were cultivated in test medium (0.1% FBS DMEM supplemented with γ-T3 or α-Toc) for 3 and 6 h. Then, mRNA levels of COX-2, IL-1β, IL-6, and MCP-1 were measured by real time RT-PCR. Data are expressed as the percentage relative to nonirradiated control cells. Values are mean ± SD (n = 6). Means without a common letter differ, $P < 0.05$.

α-Toc did not affect PGE_2 production (**Figure 2**). This result indicates the possibility that γ-T3 has a more potent anti-inflammatory effect than that of α-Toc, and may suggest that T3's unsaturated side chain structure and number of methyl group of chroman ring are important for its anti-inflammatory action.

COX-2 is one of the key enzymes required for the synthesis of PGE_2 from arachidonic acid (*30*). COX-2 plays key roles in acute inflammatory pathways in UVB-irradiated keratinocytes (*31*). Therefore, for anti-inflammatory purpose, it has been desired to suppress COX-2 expression in UVB-irradiated skin. In this study, we evaluated the effect of γ-T3 on COX-2 expression, and demonstrated that γ-T3 effectively suppresses UVB-induced mRNA and protein expression of COX-2 (**Figures 3** and **4A**). Similar to the present result, T3 reportedly suppresses TNF-α-induced COX-2 expression in human chronic myeloid leukemia cells (*32*) and lipopolysaccharide-induced COX-2 expression in human macrophage cells (*33*). This knowledge, together with our previous (*34*) and present findings (**Figure 3**), indicates that T3 may inhibit external stimulation-induced COX-2 expression. Because COX-2 inhibitors (e.g., celecoxib and NS-398) are known to reduce skin inflammation and production of inflammatory mediators (*35*), it would be possible that γ-T3 acts as an anti-inflammatory agent by inhibiting COX-2-dependent PGE_2 production. On the other hand, UVB potently induces the release of pro-inflammatory mediators such as IL-1β, IL-6, and MCP-1 from keratinocytes (**Figure 3**). These inflammatory mediators are considered to be responsible for the onset of inflammation and induction of neutrophil and macrophage chemotaxis into the skin (*36*, *37*). So, we evaluated the effect of γ-T3 and α-Toc on UVB-induced mRNA expression of IL-1β, IL-6, and MCP-1 in HaCaT cells. UVB enhanced the expression of these inflammatory genes for 3 and 6 h, and treatment with γ-T3 (1 μM) significantly suppressed these expressions. In contrast, with the exception of MCP-1, α-Toc had virtually no suppressive effect (**Figure 3**). Overall, T3 appears to mediate its anti-inflammatory action by inhibiting COX-2 and inflammatory cytokine expression (IL-1β, IL-6, and MCP-1). Next, we thought about whether γ-T3 affects upstream signal pathways that control expression of these inflammatory mediators.

Recently, the expression of inflammatory cytokines (e.g., COX-2 and IL-6) was shown to be affected by various intracellular signaling

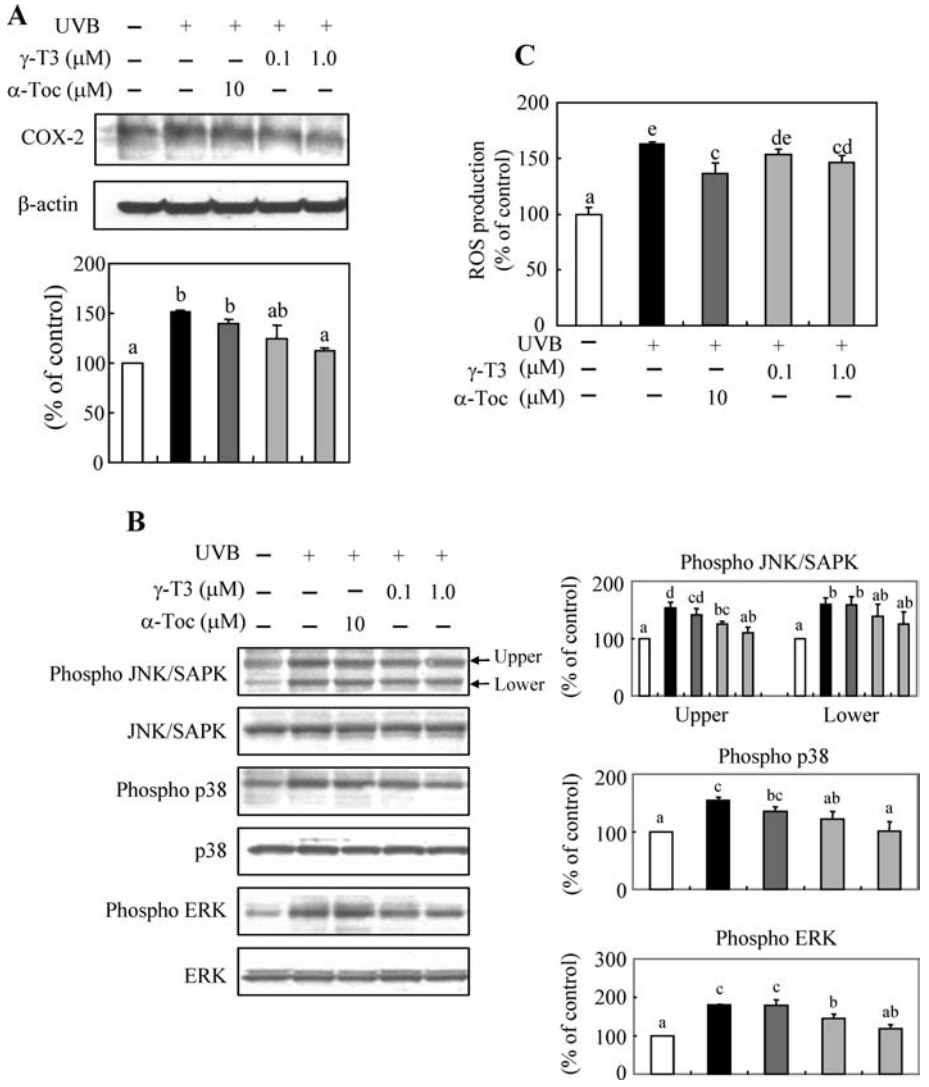


Figure 4. Effect of γ-T3 or α-Toc on UVB-induced COX-2 protein expression (**A**), MAPK protein phosphorylation (**B**), ROS generation (**C**). UVB-treated or untreated cells were cultivated in test medium (0.1% FBS DMEM supplemented with γ-T3 or α-Toc) for 24 h for Western blot analysis of COX-2 (**A**), or 2 h for Western blot analysis of JNK/SAPK, p38, and ERK (**B**). Each Western blot is a representative example of data from 3 replicate experiments. Values are mean ± SD ($n = 3$). Means without a common letter differ, $P < 0.05$. For evaluation of ROS (**C**), UVB-treated or untreated cells were cultivated in test medium (0.1% FBS DMEM supplemented with γ-T3 or α-Toc) for 3 h. The medium was then changed to sample-free test medium (0.1% FBS DMEM) containing 10 μM DCDHF-DA, followed by incubation for 30 min. Data are expressed as the percentage relative to nonirradiated control cells. Values are mean ± SD ($n = 6$). Means without a common letter differ, $P < 0.05$.

MAPK proteins such as JNK/SAPK, p38, and ERK (*38*). MAPK is involved in many cellular processes including inflammation, proliferation, differentiation, cell cycle regulation, and apoptosis (*5*, *39*). Various stressors, such as UV radiation, oxidant injury, heat shock, cytokines, and other pro-inflammatory stimuli, trigger the activation of these MAPK-dependent signaling cascades (*40*, *41*). Recently, Chen et al. reported that acute UVB exposure induces the rapid activation of MAPK signals such as p38 in HaCaT cells, which leads to COX-2 expression (*42*). In this study, we confirmed that γ-T3 suppressed UVB-induced activation of JNK/SAPK, p38, and ERK in HaCaT cells as well as expression of the COX-2 protein (**Figure 4B**). These results indicate that γ-T3 exerts its inhibitory effect on UVB-induced inflammatory mediator expression by inhibiting MAPK signaling pathways. In inflammation, JNK/SAPK, p38, and ERK are known to be involved in activating the transcription factors activator protein-1 (AP-1) and nuclear factor kappa B (NF-κB) (*43*). Inhibition of UVB-induced phosphorylation of MAPK proteins by γ-T3 may, in turn, inhibit downstream events, such as activation of AP-1 and NF-κB that can contribute to inflammatory mediator production. JNK/SAPK is known to regulate AP-1 transcription in response to environmental stresses, such as UVB exposure (*44*). Increased AP-1 activity is implicated in the promotion and progression of inflammatory reactions in HaCaT cells (*45*), while the MAPK family proteins ERK and p38 can modulate NF-κB activation (*46*). Therefore, inhibition of JNK/SAPK, ERK, and p38 activation is considered to be a molecular target for potential anti-inflammatory agents. Our results suggest that γ-T3 inhibition of UVB-induced phosphorylation of MAPKs provides an inhibitory effect on the activation of transcription factors AP-1 and NF-κB. More recent studies showed that γ-T3 inhibits NF-κB activation in cancer cells (*32*). Therefore, we consider that γ-T3 targets AP-1 and NF-κB pathways by way of MAPK to produce an inhibitory effect on inflammation.

Exposure of mammalian cells to UVB stimulates signaling pathways that activate NF-κB via ROS production (*47*). This ROS-induced NF-κB activation is caused by IκB-degradation and subsequent NF-κB nuclear localization (*48*). Furthermore,

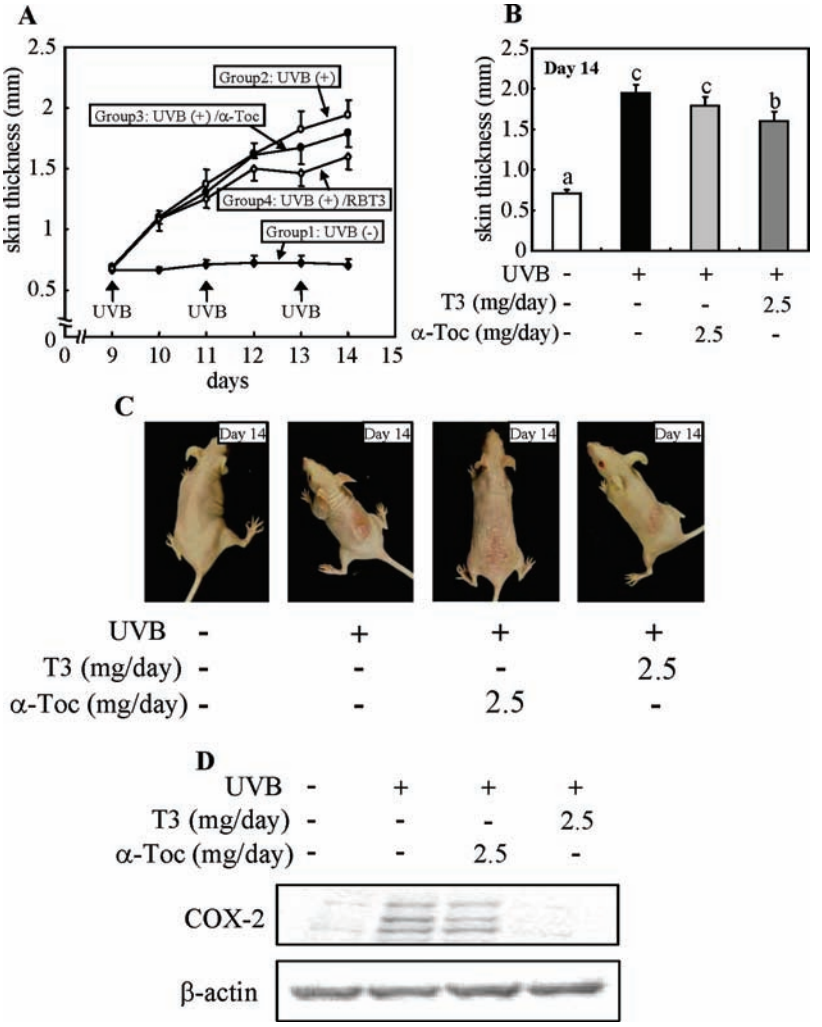


Figure 5. Effect of oral administration of RBT3 or α-Toc on skin thickening (**A**, **B**), sunburn reaction (**C**), and COX-2 protein expression (**D**) in UVB-irradiated HR-1 hairless mice. The mice were divided into 4 groups: group 1, untreated/vehicle (Toc-stripped corn oil); group 2, UVB-irradiated/vehicle; group 3, UVB-irradiated/2.5 mg of α-Toc/day; and group 4, UVB-irradiated/2.57 mg of RBT3 (0.09 mg of α-T3, 2.31 mg of γ-T3, 0.1 mg of δ-T3, 0.04 mg of γ-Toc, and 0.02 mg of δ-Toc)/day. Each mouse received samples (dissolved in Toc-stripped corn oil and total 50 mg) orally once a day for 14 successive days. On days 9, 11, and 13, dorsal mouse skin was exposed to UVB irradiation at a dose of 200 mJ/cm^2. From day 9 to day 14, skin thickness was measured daily at three midline sites using a caliper (**A**). The data of skin thickness on day 14 is presented (**B**). Values are mean ± SD ($n = 8$). Means without a common letter differ, $P < 0.05$. Each picture is a representative example of picture on day 14 (**C**). On day 14, skins were collected for Western blot analysis of COX-2 protein (**D**). Each Western blot is a representative example of the data.

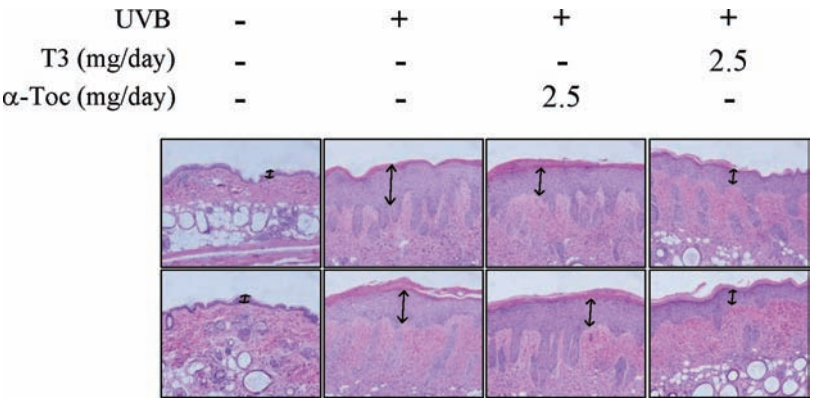


Figure 6. Effect of oral administration of RBT3 or α-Toc on histology of UVB-irradiated HR-1 hairless mouse skin. Skin samples were fixed in 4% formalin, and embedded in paraffin. Vertical sections (5 μm) were cut, and stained with hematoxylin and eosin. Arrow represents epidermal layer thickness. Each figure is a representative example of data.

UV-generated ROS can also activate diverse downstream MAPK signaling molecules such as JNK/SAPK and p38 MAPK (*49*). Our results showed that both γ-T3 and α-Toc suppressed UVB-induced ROS production (**Figure 4C**). This indicates that suppression

Table 1. Tissue Concentrations of T3 and Toc in Skin

	skin (nmol/g)			
	group 1 (control)	group 2 (UVB)	group 3 (UVB/α-Toc)	group 4 (UVB/RBT3)
T3 total	0.2 ± 0.1[a]	0.2 ± 0.1	0.1 ± 0.1	70.5 ± 31.4
α-T3	0.2 ± 0.1	0.2 ± 0.1	0.1 ± 0.1	5.6 ± 1.9
β-T3	nd[b]	nd	nd	1.3 ± 0.5
γ-T3	nd	nd	nd	62.4 ± 28.3
δ-T3	nd	nd	nd	1.2 ± 0.9
Toc total	7.0 ± 2.4[a]	5.3 ± 0.7	39.6 ± 8.6	8.9 ± 2.6
α-Toc	7.0 ± 2.4[a]	5.3 ± 0.7	39.6 ± 8.6	8.9 ± 2.6
β-Toc	nd	nd	nd	nd
γ -Toc	nd	nd	nd	nd
δ-Toc	nd	nd	nd	nd

[a] Values represent mean ± SD. [b] Not detectable.

of MAPK activation by γ-T3 is based not only on the direct inhibitory effect on cellular signaling but also on inhibition of ROS production. A study to further elucidate the detailed mechanism of T3's anti-inflammatory effect is ongoing.

Finally, to evaluate the *in vivo* effect of T3 or α-Toc on UVB-induced skin inflammation, we conducted a skin thickening assay using HR-1 hairless mice. Many studies demonstrated the usefulness of this assay to assess the *in vivo* efficacy of compounds for preventing photodamage in skin (*5*−*7*, *11*). As shown in **Figure 5C**, severe sunburn was observed in the UVB-irradiated control group. The group fed RBT3 showed comparatively slight sunburn. UVB prompted the increase in skin thickness that could be attenuated by oral administration of RBT3 (**Figures 5A** and **5B**). In addition, feeding RBT3 suppressed UVB-induced COX-2 expression (**Figure 5D**) as well as epidermal hyperplasia (**Figure 6**). Therefore, T3 likely acts as an anti-inflammatory agent mainly by inhibiting COX-2 expression *in vivo*. Inhibition of COX-2 expression has also been shown to be an important mechanism of action for other anti-inflammatory compounds besides T3, including epigallocatechin gallate (*5*), resveratrol (*6*), and curcumin (*45*). In our animal study, a protective effect of α-Toc against skin damage was observed, but its effect was not significant (**Figures 5** and **6**). Our results were thus consistent with the report by Yamada et al. that T3 attenuates UVB-induced skin damage in hairless mice (α-Toc had little effect in this study as well) (*11*). Ikeda et al. reported that substantial amounts of α-T3 and γ-T3 were detected in rat and mouse skin whereas T3 levels were very low in the liver, kidney and plasma (*50*). In our results, feeding RBT3 resulted in remarkably high levels of γ-T3 in the skin of hairless mice (62.4 ± 28.3 nmol/g skin), with γ-T3 accumulating in the skin more easily than α-Toc (39.6 ± 8.6 nmol/g skin) even when the same quantities of γ-T3 and α-Toc were administered (**Table 1**). Similar to the present result, it was reported that T3 is more easily incorporated into cultured cells than Toc (*51*). These points may explain why dietary γ-T3 is a better protective compound against UVB-induced skin damage than α-Toc.

In conclusion, we evaluated the effect of γ-T3 and α-Toc on skin inflammation *in vitro* and *in vivo*, and found that T3 has a potent ability to attenuate UVB-induced skin inflammation by suppressing MAPK activation and inhibiting COX-2 expression. Our findings provide a new insight into the nutritional or pharmacological applications of T3.

ABBREVIATIONS USED

AP-1, activator protein-1; COX-2, cyclooxgenase-2; DCDHF-DA, 2,7-dichlorodihydrofluorescein diacetate; ELISA, enzyme-linked immunosorbent assay; ERK, extracellular signal regulated kinase; FBS, fetal bovine serum; FL-HPLC, fluorescence HPLC; HaCaT, immortalized human keratinocytes; IL, interleukin; JNK/SAPK, c-Jun N-terminal kinase/stress-activated protein kinase; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemotactic protein-1; NF-κB, nuclear factor kappa B; PGs, prostaglandins; PGE_2, prostaglandin E_2; ROS, reactive oxygen species; RBT3, rice bran tocotrienol; RT-PCR, reverse transcription-PCR; SDS−PAGE, sodium dodecyl sulfate−polyacrylaminde gel electrophoresis; T3, tocotrienol; Toc, tocopherol; UV, ultraviolet.

ACKNOWLEDGMENT

We thank Prof. Norimichi Nakahata (Graduate School of Pharmaceutical Sciences, Tohoku University, Japan) for the donation of HaCaT.

Supporting Information Available: Table of primer sequences for cDNA amplification of selected human genes. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Bruls, W. A. G.; Vanweelden, H.; Vanderleun, J. C. Transmission of UV-radiation through human epidermal layers as a factor influencing the minimal erythema dose. *Photochem. Photobiol.* **1984**, *39*, 63–67.
(2) El-Ghorr, A. A.; Norval, M. Biological effects of narrow-band (311 nm TL01) UVB irradiation: a review. *J. Photochem. Photobiol. B* **1997**, *38*, 99–106.
(3) Clydesdale, G. J.; Dandie, G. W.; Muller, H. K. Ultraviolet light induced injury: immunological and inflammatory effects. *Immunol. Cell Biol.* **2001**, *79*, 547–568.
(4) Pupe, A.; Moison, R.; De Haes, P.; van Henegouwen, G. B.; Rhodes, L.; Degreef, H.; Garmyn, M. Eicosapentaenoic acid, a n-3 polyunsaturated fatty acid differentially modulates TNF-α, IL-1α, IL-6 and PGE2 expression in UVB-irradiated human keratinocytes. *J. Invest. Dermatol.* **2002**, *118*, 692–698.
(5) Vayalil, P. K.; Elmets, C. A.; Katiyar, S. K. Treatment of green tea polyphenols in hydrophilic cream prevents UVB-induced oxidation of lipids and proteins, depletion of antioxidant enzymes and phosphorylation of MAPK proteins in SKH-1 hairless mouse skin. *Carcinogenesis* **2003**, *24*, 927–936.
(6) Afaq, F.; Adhami, V. M.; Ahmad, N. Prevention of short-term ultraviolet B radiation-mediated damages by resveratrol in SKH-1 hairless mice. *Toxicol. Appl. Pharmacol.* **2003**, *186*, 28–37.
(7) Shibata, A.; Nakagawa, K.; Yamanoi, H.; Tsuduki, T.; Sookwong, P.; Higuchi, O.; Kimura, F.; Miyazawa, T. Sulforaphane suppresses ultraviolet B-induced inflammation in HaCaT keratinocytes and HR-1 hairless mice. *J. Nutr. Biochem.* **2009**.
(8) Talalay, P.; Fahey, J. W.; Healy, Z. R.; Wehage, S. L.; Benedict, A. L.; Min, C.; Dinkova-Kostova, A. T. Sulforaphane mobilizes cellular defenses that protect skin against damage by UV radiation. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 17500–17505.
(9) Maalouf, S.; El-Sabban, M.; Darwiche, N.; Gali-Muhtasib, H. Protective effect of vitamin E on ultraviolet B light-induced damage in keratinocytes. *Mol. Carcinog.* **2002**, *34*, 121–130.
(10) Burke, K. E.; Clive, J.; Combs, G. F.; Commisso, J.; Keen, C. L.; Nakamura, R. M. Effects of topical and oral vitamin E on pigmentation and skin cancer induced by ultraviolet irradiation in Skh: 2 hairless mice. *Nutr. Cancer* **2000**, *38*, 87–97.
(11) Yamada, Y.; Obayashi, M.; Ishikawa, T.; Kiso, Y.; Ono, Y.; Yamashita, K. Dietary tocotrienol reduces UVB-induced skin damage and sesamin enhances tocotrienol effects in hairless mice. *J. Nutr. Sci. Vitaminol.* (*Tokyo*) **2008**, *54*, 117–123.
(12) Yoshida, E.; Watanabe, T.; Takata, J.; Yamazaki, A.; Karube, Y.; Kobayashi, S. Topical application of a novel, hydrophilic γ-tocopherol derivative reduces photo-inflammation in mice skin. *J. Invest. Dermatol.* **2006**, *126*, 1633–1640.
(13) Kawakami, Y.; Tsuzuki, T.; Nakagawa, K.; Miyazawa, T. Distribution of tocotrienols in rats fed a rice bran tocotrienol concentrate. *Biosci., Biotechnol., Biochem.* **2007**, *71*, 464–471.
(14) Boukamp, P.; Petrussevska, R. T.; Breitkreutz, D.; Hornung, J.; Markham, A.; Fusenig, N. E. Normal keratinization in a spontaneously

immortalized aneuploid human keratinocyte cell-line. *J. Cell Biol.* **1988**, *106*, 761–771.
(15) Ishiyama, M.; Tominaga, H.; Shiga, M.; Sasamoto, K.; Ohkura, Y.; Ueno, K. A combined assay of cell viability and in vitro cytotoxicity with a highly water-soluble tetrazolium salt, neutral red and crystal violet. *Biol. Pharm. Bull.* **1996**, *19*, 1518–1520.
(16) Nakagawa, K.; Shibata, A.; Yamashita, S.; Tsuzuki, T.; Kariya, J.; Oikawa, S.; Miyazawa, T. In vivo angiogenesis is suppressed by unsaturated vitamin E, tocotrienol. *J. Nutr.* **2007**, *137*, 1938–1943.
(17) Jeon, K. S.; Na, H. J.; Kim, Y. M.; Kwon, H. J. Antiangiogenic activity of 4-*O*-methylgallic acid from Canavalia gladiata, a dietary legume. *Biochem. Biophys. Res. Commun.* **2005**, *330*, 1268–1274.
(18) Tiidus, P. M.; Houston, M. E. Vitamin E status does not affect the responses to exercise training and acute exercise in female rats. *J. Nutr.* **1993**, *123*, 834–840.
(19) Shibata, A.; Nakagawa, K.; Sookwong, P.; Tsuzuki, T.; Oikawa, S.; Miyazawa, T. Tumor anti-angiogenic effect and mechanism of action of δ-tocotrienol. *Biochem. Pharmacol.* **2008**, *76*, 330–339.
(20) Shibata, A.; Nakagawa, K.; Sookwong, P.; Tsuduki, T.; Oikawa, S.; Miyazawa, T. δ-Tocotrienol suppresses VEGF induced angiogenesis whereas α-tocopherol does not. *J. Agric. Food Chem.* **2009**, *57*, 8696–8704.
(21) Grone, A. Keratinocytes and cytokines. *Vet. Immunol. Immunopathol.* **2002**, *88*, 1–12.
(22) Rhodes, L. E.; Durham, B. H.; Fraser, W. D.; Friedmann, P. S. Dietary fish oil reduces basal and ultraviolet B-generated PGE2 levels in skin and increases the threshold to provocation of polymorphic light eruption. *J. Invest. Dermatol.* **1995**, *105*, 532–535.
(23) Kaminska, B. MAPK signalling pathways as molecular targets for anti-inflammatory therapy - from molecular mechanisms to therapeutic benefits. *Biochim. Biophys. Acta* **2005**, *1754*, 253–262.
(24) Thannickal, V. J.; Fanburg, B. L. Reactive oxygen species in cell signaling. *Am. J. Physiol.* **2000**, *279*, L1005–1028.
(25) Matsumura, Y.; Ananthaswamy, H. N. Toxic effects of ultraviolet radiation on the skin. *Toxicol. Appl. Pharmacol.* **2004**, *195*, 298–308.
(26) Yaar, M.; Gilchrest, B. A. Photoageing: mechanism, prevention and therapy. *Br. J. Dermatol.* **2007**, *157*, 874–887.
(27) Storey, A.; Rogers, J. S.; McArdle, F.; Jackson, M. J.; Rhodes, L. E. Conjugated linoleic acids modulate UVR-induced IL-8 and PGE2 in human skin cells: potential of CLA isomers in nutritional photoprotection. *Carcinogenesis* **2007**, *28*, 1329–1333.
(28) Hinson, R. M.; Williams, J. A.; Shacter, E. Elevated interleukin 6 is induced by prostaglandin E2 in a murine model of inflammation: possible role of cyclooxygenase-2. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 4885–4890.
(29) Yu, Y.; Chadee, K. Prostaglandin E2 stimulates IL-8 gene expression in human colonic epithelial cells by a posttranscriptional mechanism. *J. Immunol.* **1998**, *161*, 3746–3752.
(30) Black, A. T.; Gray, J. P.; Shakarjian, M. P.; Mishin, V.; Laskin, D. L.; Heck, D. E.; Laskin, J. D. UVB light upregulates prostaglandin synthases and prostaglandin receptors in mouse keratinocytes. *Toxicol. Appl. Pharmacol.* **2008**, *232*, 14–24.
(31) Isoherranen, K.; Punnonen, K.; Jansen, C.; Uotila, P. Ultraviolet irradiation induces cyclooxygenase-2 expression in keratinocytes. *Br. J. Dermatol.* **1999**, *140*, 1017–1022.
(32) Ahn, K. S.; Sethi, G.; Krishnan, K.; Aggarwal, B. B. γ-tocotrienol inhibits nuclear factor-κB signaling pathway through inhibition of receptor-interacting protein and TAK1 leading to suppression of antiapoptotic gene products and potentiation of apoptosis. *J. Biol. Chem.* **2007**, *282*, 809–820.
(33) Yam, M. L.; Abdul Hafid, S. R.; Cheng, H. M.; Nesaretnam, K. Tocotrienols suppress proinflammatory markers and cyclooxygenase-2 expression in RAW264.7 macrophages. *Lipids* **2009**, *44*, 787–797.
(34) Shibata, A.; Nakagawa, K.; Sookwong, P.; Tsuduki, T.; Tomita, S.; Shirakawa, H.; Komai, M.; Miyazawa, T. Tocotrienol inhibits secretion of angiogenic factors from human colorectal adenocarcinoma cells by suppressing hypoxia-inducible factor-1α. *J. Nutr.* **2008**, *138*, 2136–2142.
(35) Tipton, D. A.; Flynn, J. C.; Stein, S. H.; Dabbous, M. Cyclooxygenase-2 inhibitors decrease interleukin-1β-stimulated prostaglandin E2 and IL-6 production by human gingival fibroblasts. *J. Periodontol.* **2003**, *74*, 1754–1763.
(36) Urbanski, A.; Schwarz, T.; Neuner, P.; Krutmann, J.; Kirnbauer, R.; Kock, A.; Luger, T. A. Ultraviolet light induces increased circulating interleukin-6 in humans. *J. Invest. Dermatol.* **1990**, *94*, 808–811.
(37) Yano, S.; Banno, T.; Walsh, R.; Blumenberg, M. Transcriptional responses of human epidermal keratinocytes to cytokine interleukin-1. *J. Cell Physiol.* **2008**, *214*, 1–13.
(38) Ashida, M.; Bito, T.; Budiyanto, A.; Ichihashi, M.; Ueda, M. Involvement of EGF receptor activation in the induction of cyclooxygenase-2 in HaCaT keratinocytes after UVB. *Exp. Dermatol.* **2003**, *12*, 445–452.
(39) Cuevas, B. D.; Abell, A. N.; Johnson, G. L. Role of mitogen-activated protein kinase kinase kinases in signal integration. *Oncogene* **2007**, *26*, 3159–3171.
(40) Chang, L.; Karin, M. Mammalian MAP kinase signalling cascades. *Nature* **2001**, *410*, 37–40.
(41) Kyriakis, J. M.; Avruch, J. Mammalian mitogen-activated protein kinase signal transduction pathways activated by stress and inflammation. *Physiol. Rev.* **2001**, *81*, 807–869.
(42) Chen, W.; Tang, Q.; Gonzales, M. S.; Bowden, G. T. Role of p38 MAP kinases and ERK in mediating ultraviolet-B induced cyclooxygenase-2 gene expression in human keratinocytes. *Oncogene* **2001**, *20*, 3921–3926.
(43) Whitmarsh, A. J.; Davis, R. J. Transcription factor AP-1 regulation by mitogen-activated protein kinase signal transduction pathways. *J. Mol. Med.* **1996**, *74*, 589–607.
(44) Koj, A. Initiation of acute phase response and synthesis of cytokines. *Biochim. Biophys. Acta* **1996**, *1317*, 84–94.
(45) Cho, J. W.; Park, K.; Kweon, G. R.; Jang, B. C.; Baek, W. K.; Suh, M. H.; Kim, C. W.; Lee, K. S.; Suh, S. I. 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. *Exp. Mol. Med.* **2005**, *37*, 186–192.
(46) Cho, J. W.; Lee, K. S.; Kim, C. W. Curcumin attenuates the expression of IL-1beta, IL-6, and TNF-α as well as cyclin E in TNF-α-treated HaCaT cells; NF-κB and MAPKs as potential upstream targets. *Int. J. Mol. Med.* **2007**, *19*, 469–474.
(47) Beak, S. M.; Lee, Y. S.; Kim, J. A. NADPH oxidase and cyclooxygenase mediate the ultraviolet B-induced generation of reactive oxygen species and activation of nuclear factor-κB in HaCaT human keratinocytes. *Biochimie* **2004**, *86*, 425–429.
(48) Piette, J.; Piret, B.; Bonizzi, G.; Schoonbroodt, S.; Merville, M. P.; Legrand-Poels, S.; Bours, V. Multiple redox regulation in NF-κB transcription factor activation. *Biol. Chem.* **1997**, *378*, 1237–1245.
(49) Van Laethem, A.; Nys, K.; Van Kelst, S.; Claerhout, S.; Ichijo, H.; Vandenheede, J. R.; Garmyn, M.; Agostinis, P. Apoptosis signal regulating kinase-1 connects reactive oxygen species to p38 MAPK-induced mitochondrial apoptosis in UVB-irradiated human keratinocytes. *Free Radical Biol. Med.* **2006**, *41*, 1361–1371.
(50) Ikeda, S.; Niwa, T.; Yamashita, K. Selective uptake of dietary tocotrienols into rat skin. *J. Nutr. Sci. Vitaminol. (Tokyo)* **2000**, *46*, 141–143.
(51) Saito, Y.; Yoshida, Y.; Nishio, K.; Hayakawa, M.; Niki, E. Characterization of cellular uptake and distribution of vitamin E. *Ann. N.Y. Acad. Sci.* **2004**, *1031*, 368–375.

Received for review February 19, 2010. Revised manuscript received April 7, 2010. Accepted April 27, 2010.