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Prevention of UVB-induced photoinflammation and photoaging by a polymethoxy flavonoid, nobiletin, in human keratinocytes in vivo and in vitro

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Abstract

Exposure to ultraviolet B (UVB) irradiation induces acute skin inflammation such as erythema (sunburn) and edema, and prostaglandin (PG)E_2 in the epidermis plays an important role as its prominent mediator. In the present study, we investigated the effect of nobiletin (5,6,7,8,3′,4′-hexamethoxy flavone) from *Citrus depressa*, on the production of PGE_2 in UVB-irradiated human keratinocytes. When keratinocytes were irradiated with 60 mJ of UVB/cm^2, the production and gene expression of cyclooxygenase (COX)-2, but not COX-1, were augmented along with an increase in PGE_2 levels. The augmented COX-2 production was transcriptionally suppressed by nobiletin. In addition, neither the release of [^{14}C]arachidonic acid from membrane phospholipids nor the gene expression of cytosolic phospholipase A_2 ($cPLA_2$) was altered in UVB-irradiated human keratinocytes. However, nobiletin was found to inhibit the release of [^{14}C]arachidonic acid by decreasing the Ca^{2+}-dependent activity of $cPLA_2$. Furthermore, topical treatment of nobiletin on the skin of the back prevented the UVB-induced increase of transepidermal water loss and hyperplasia of the epidermis in hairless mice. Therefore, these results suggest that nobiletin inhibits the UVB-induced production of PGE_2 not only by suppressing the expression of COX-2 but also by decreasing the activity of $cPLA_2$ in human keratinocytes. Furthermore, nobiletin may be useful as a novel sunscreen reagent to be applied for protection against photoinflammation and photoaging.

Keywords: Nobiletin; Ultraviolet B; Photoinflammation; Cyclooxygenase; Cytosolic phospholipase A_2; Human keratinocytes

1. Introduction

Ultraviolet B (UVB) irradiation of the skin induces acute inflammation, characterized by erythema, edema and immunosuppression, and is subsequently linked to the progression of skin cancer [1–3]. The most prominent visible sign of UVB inflammation in human skin is erythema, the primary mediator of which is prostaglandin E_2 (PGE_2) [4–7]. It has also been reported that UVB-induced PGE_2 production is due to the augmented expression of cyclooxygenase (COX)-2 in the epidermis [8–12], the tissue that absorbs the greater part of incident UVB radiation [1,13]. In addition, UVB has been reported to stimulate human skin and keratinocytes to augment the synthesis and activation of cytosolic phospholipase A_2 ($cPLA_2$) [14], which in turn increases the release of arachidonic acid from membrane phospholipids [15]. Therefore, it is likely that UVB-induced erythema involves the augmented synthesis and activation of both $cPLA_2$ and COX-2 in the epidermis.

Sunscreen is an efficient strategy for preventing acute and chronic UV damage in skin such as erythema, photoaging and photocarcinogenesis [2,16], and therefore physiological and pharmacological candidates have been identified for the development of possible novel skin care products and chemopreventive agents [17–19]. Bissett et al.

Abbreviations: UVB, ultraviolet B; PG, prostaglandin; COX, cyclooxygenase; $cPLA_2$, cytosolic phospholipase A_2; MAFP, methyl arachidonyl pyrocarbonate; RT-PCR, reverse transcriptase-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TEWL, transepidermal water loss

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[20] have reported that the physiologic superoxide-scavenging antioxidants, α-tocopherol and ascorbic acid, reduce UVB-induced photoaging in the skin of albino hairless mice. In addition, pentoxifylline, a methylxanthine derivative, has been reported to prevent sunburn and erythema in human skin due to its anti-oxidant activity [21,22]. On the other hand, flavonoids from medicinal plants possess various pharmacological effects such as anti-inflammatory, anti-oxidant, and radical scavenging activities [23–25]. As far as photoprotection by flavonoids is concerned, silymarin and genistein have been found to be effective in preventing UV-induced immunosuppression and inflammation since they decrease oxidative stress [12,26]. Recently, we reported that nobiletin, a polymethoxy flavonoid from *Citrus depressa*, inhibits the production of PGE_2 in articular chondrocytes and synovial fibroblasts from rabbits and humans [27,28]. In addition, nobiletin has been found to suppress the expression of proinflammatory cytokines such as interleukin-1, interleukin-6, and tumor necrosis factor-α in mouse macrophages [28]. Furthermore, nobiletin inhibits tumor invasiveness and progression in vivo and in vitro [29–31]. Therefore, nobiletin may be a candidate for a novel sunscreen product that prevents UVB-induced acute and chronic skin damage. However, it has not yet been clarified whether nobiletin may modulate UVB-induced inflammatory responses.

In the present study, we demonstrated that nobiletin inhibited the UVB-induced production of PGE_2 due to the suppression of COX-2 expression in human keratinocytes. In addition, nobiletin suppressed the release of arachidonic acid from membrane phospholipids by inhibiting the activity of $cPLA_2$ but not its production, suggesting that nobiletin is a novel anti-photoinflammation reagent for the prevention of acute skin damage such as erythema and edema.

2. Materials and methods

2.1. Cell culture

Human epidermal keratinocytes (Clonetics, San Diego, CA) were placed on 24- and 96-well multiplates, 60 mm dishes or 100 mm dishes coated with type-I collagen (3 μg/ml; Nitta Gelatin, Osaka, Japan) and cultured in KGM medium supplemented with bovine pituitary extract, recombinant human epidermal growth factor (0.1 ng/ml), insulin (5.0 μg/ml), hydrocortisone (0.5 μg/ml), gentamicin (50 μg/ml) and amphotericin-B (50 ng/ml) (Clonetics) until confluence as previously described [32]. In this series of experiments, the keratinocytes used were in the 2nd–4th passage.

2.2. UV irradiation and cell treatments

Confluent human keratinocytes were exposed to UVB irradiation using a fluorescent FL20S sunlamp (280 and 400 nm, a peak emission of 313 nm) (Toshiba, Tokyo, Japan) at a distance of 30 cm. Lamp output was measured using an MI-340 light meter (Eko, Tokyo, Japan) with a MS-330B sensor for UVB (280–320 nm) and a MS-330A sensor for UVA (320–400 nm). The average irradiance in this system was 2.0 W/m^2 UVB (280–320 nm), 0.9 W/m^2 UVAII (320–340 nm) and 0.8 W/m^2 UVAI (340–400 nm). The total dose of UVB exposed to the cells was 30–60 mJ/cm^2, which could be normally received by skin from sunlight. Sham-irradiated cultures (controls) were similarly handled, except that they were shielded with aluminum foil during the irradiation. For nobiletin or COX inhibitors plus UVB treatments, the cells were pre-treated with nobiletin (5,6,7,8,3′,4′-hexamethoxy flavone) that was isolated from the juice of *C. depressa* "Hayata" [27], and COX inhibitors: indomethacin, aspirin (Sigma Chemical Co., St. Louis, MO), and NS-398 (Calbiochem-Novabiochem, San Diego, CA) [33,34] for 30 min, and then irradiated with UVB. After UVB irradiation, the cell treatments were continued without changing the culture medium until harvest. Nobiletin and COX inhibitors were added to the culture medium as a dimethyl sulfoxide (DMSO) solution. The final DMSO concentration was 0.05% in all cultures, and the same vehicle amount was added to the control cultures.

2.3. PGE_2 measurement

PGE_2 contents in the culture media were measured by radioimmunoassay as described previously [32,35]. Briefly, an aliquot (50 μl) of the harvested culture medium was incubated with [^{3}H]PGE_2 (DuPont NEN, Boston, MA) and PGE_2 antibody (PerSeptive Diagnostics, Cambridge, MA) in a total volume of 160 μl for 18 h at 4 °C and then 500 μl of 0.025% dextran/0.25% charcoal/0.9% NaCl were added to remove the unbound [^{3}H]PGE_2. After incubation for 15 min at 4 °C, the reaction mixture was subjected to centrifugation and the radioactivity in the resultant supernatant was counted. The amount of PGE_2 was calculated from a standard curve performed concomitantly using authentic PGE_2. Otherwise, the levels of PGE_2 in the culture media were measured by a PGE_2 enzyme immunoassay kit (Amersham Biosciences, Tokyo, Japan) according to the manufacturer's instructions.

2.4. Alamer blue assay

To evaluate the cell viability of the UVB-irradiated keratinocytes, confluent keratinocytes in 24-well multiplates coated with type-I collagen were irradiated with UVB, and then cultured for 24 h. For the last 3 h of the culture, Alamer blue (Asahi Techno Glass, Tokyo, Japan) was added to the cells and then the absorbance of the incorporated reagent was measured at 590 nm as previously described [28,36].

2.5. Release of [^{14}C]arachidonic acid from membrane phospholipids

Confluent human keratinocytes were labeled with [^{14}C]arachidonic acid (Amersham Biosciences) (3.7 kBq/ml) for 24 h as previously described [35]. After washing once with the culture medium, the cells were subjected to UVB irradiation in the presence or absence of nobiletin, and then cultured for up to 8 h. In addition, [^{14}C]arachidonic-acid-labeled keratinocytes were treated with a calcium ionophore, A23187 (Calbiochem-Novabiochem), in the presence or absence of nobiletin and a $cPLA_2$ inhibitor, methyl arachidonyl pyrocarbonate (MAFP) (Calbiochem-Novabiochem) for 8 h. The radioactivity of [^{14}C]arachidonic acid in the harvested culture media was determined by scintillation counting. MAFP was added to the culture medium as a DMSO solution (the final concentration: 0.1%) and the same vehicle amount was added to the control cultures.

2.6. Semiquantification of COX-1, COX-2 and $cPLA_2$ mRNA levels by RT-PCR

Gene expression of COX-1, COX-2 and $cPLA_2$ in the UVB cells was monitored by reverse transcriptase-polymerase chain reaction (RT-PCR) as previously described [28]. Briefly, isolated RNA (1 μg) was subjected to first-strand cDNA synthesis and then one-tenth of the generated cDNA was used for PCR amplification. The PCR primers used were the following: human COX-1: 5′-TGCCCAGCTCCTGGCCCGCCGCTT-3′ (sense) (516–539 bp) and 5′-GTGCATCAACACAGGCGCCTCTTC-3′ (antisense) (796–819 bp) [37], human COX-2: 5′-TTCAAATGAGATTGTGGGAAAAT-3′ (sense) (574–596 bp) and 5′-AGATCATCTCTGCCTGAGTATCTT-3′ (antisense) (855–878 bp) [8], human $cPLA_2$: 5′-GAGCTGATGTTTGCAGATTGGGTTG-3′ (sense) (1156–1180 bp) and 5′-GTCACTCAAAGGAGACAGTGGATAATA-3′ (antisense) (1639–1665 bp) [38]and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH): 5′-CCACCCATGGCAAATTCCATGGCA-3′ (sense) (213–235 bp) and 5′-TCTAGACGGCAGGTCAGGTCCACC-3′ (antisense) (786–809 bp) [39]. The amplification was performed at 92 °C for 40 s, 54 °C for 40 s and 72 °C for 60 s between 30 and 38 cycles after initial denaturation at 94 °C for 2 min. The amplified PCR products of COX-1 (304 bp), COX-2 (305 bp), $cPLA_2$ (510 bp) and GAPDH (456 bp) were analyzed with 1% agarose gel and visualized by ethidium bromide staining. We confirmed the cDNA sequences of the amplified $cPLA_2$ after TA cloning using pGEM-T vectors (Promega, Madison, WI) as previously described [31,35]. COX-1, COX-2, $cPLA_2$ and GAPDH gene expressions were quantified by densitometric scanning using an Image Analyzer LAS-1000 plus (Fuji Film, Tokyo, Japan). The signal intensities of the specific mRNA were normalized by comparison with that of GAPDH and calculated as relative amounts.

2.7. Transepidermal water loss and epidermal thickness in UVB-irradiated mice

For evaluating the photoprotective activity of nobiletin in vivo, transepidermal water loss (TEWL) and epidermal thickness were measured in UVB-irradiated mice. Briefly, the skin of the back of 6-week-old male hairless mice was pretreated with 100 μl of 1% nobiletin in 80% ethanol/15% 1,3-butanediol/5% glycerol every day for 7 days. After the pretreatment, the mice were irradiated with UVB (54 mJ/cm^2) every 2 days and were treated with the same amount of nobiletin for 10 days. TEWL was measured in the center of the back skin using a TEWA Meter (Courage, Khazaka, Germany). For histological examinations, the excited skin was snap-frozen in liquid nitrogen and the frozen tissues were cut into 5-μm thick sections for hematoxylin staining. Epidermal thickness in fifteen randomly chosen areas per section was measured under a light microscope at 200-fold magnification. The animals had free access to food and water according to the Guidelines of Experimental Animal Care issued by the Prime Minister's Office of Japan. The experimental protocol was approved by the Committee of Animal Care and Use of Tokyo University of Pharmacy and Life Science.

2.8. Statistical analysis

Data are presented as the mean ± S.D. and were analyzed by analysis of variance and by unpaired *t*-test. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Inhibition of UVB-induced production of PGE_2 by nobiletin in human keratinocytes

We first examined the effect of UVB irradiation on the production of PGE_2 in the cultured human keratinocytes. When the cells were irradiated with UVB at 30 and 60 mJ/cm^2 and then maintained for 24 h, a dose-dependent increase in PGE_2 levels in the culture media was observed (Table 1). In addition, an Alamer blue assay to detect cell viability under the culture conditions showed that there was no cellular toxicity to UVB at 30 and 60 mJ/cm^2 (Table 1). Therefore, we decided to employ UVB irradiation at 60 mJ/cm^2 in the following experiments.

To evaluate whether nobiletin was efficient in preventing UVB-induced inflammatory responses in the epidermis, we examined the effect of nobiletin on UVB-induced PGE_2 production in human keratinocytes. As shown in Fig. 1A, the UVB-induced levels of PGE_2 were found to decrease

Table 1
PGE_2 production and cell viability in UVB-irradiated human keratinocytes

UVB (mJ/cm^2) irradiation	PGE_2 production (pg/ml)	Cell viability (% of un-irradiated cells)
0	0.70 ± 0.06	100.00
30	0.73 ± 0.08	87.50 ± 6.25
60	1.34 ± 0.16**	92.85 ± 14.29

Confluent human keratinocytes at the 3rd passage in 24-well multiplates (for PGE_2 production) and in 96-well multiplates (for cell viability) were irradiated with UVB at 30 and 60 mJ/cm^2 and then cultured for 24 h. To measure the production of PGE_2, the harvested culture media were subjected to PGE_2 measurement by radioimmunoassay as described in Section 2. To measure the cell viability of UVB-irradiated keratinocytes, Alamer blue was incorporated into the cells for the last 3 h of culture, and then its absorbance was measured at 590 nm as described in Section 2. The data are indicated as the mean ± S.D. from four individual wells.

** Significantly different from non-irradiated cells (0 mJ/cm^2) ($P < 0.01$).

dose-dependently by nobiletin: complete inhibition was observed at 16 μM ($P < 0.05$), and at higher concentrations (32 and 64 μM) the PGE_2 levels fell below the level for untreated cells ($P < 0.001$). Basal levels of PGE_2 in untreated keratinocytes also decreased upon nobiletin treatment ($P < 0.05$). As shown in Fig. 1B, an augmentation of PGE_2 levels in culture media was observed for >6 h after UVB irradiation ($P < 0.01$). In addition, nobiletin (64 μM) was found to abolish PGE_2 levels in both untreated and UVB-irradiated keratinocytes as early as 2 h ($P < 0.01$). Furthermore, since flavonoids generally absorb UV radiation [23], we carried out treatment with nobiletin after UVB irradiation, and obtained a similar inhibition of PGE_2 production in keratinocytes (data not shown), indicating that the nobiletin-mediated suppression of PGE_2 production was independent of the general property of UV absorption of flavonoids. Thus, these results suggest that nobiletin may be a potent inhibitor of photoinflammation since it prevents the increase of PGE_2 production in UVB-irradiated keratinocytes.

3.2. Suppression of the expression of COX-2 by nobiletin in UVB-irradiated human keratinocytes

To clarify the mechanisms of the nobiletin-mediated inhibition of PGE_2 production in UVB-irradiated keratinocytes, we examined the effect of nobiletin on the expression of COXs. As shown in Fig. 2A, keratinocytes constitutively expressed COX-1 mRNA and neither UVB nor nobiletin (64 μM) altered its levels. In addition, the expression of COX-2 mRNA was induced for 6 h after UVB irradiation, and nobiletin (64 μM) was found to completely inhibit the UVB-induced COX-2 gene expression (Fig. 2B). Furthermore, we demonstrated that the UVB-induced PGE_2 production was completely inhibited by a non-selective COX inhibitor, indomethacin (10 μM) and a COX-2 specific inhibitor, NS-398 (1 μM) ($P < 0.01$, respectively). Therefore, these results suggest that the inhibition of PGE_2 by nobiletin is due to the suppression of COX-2 expression in UVB-irradiated human keratinocytes.

3.3. Inhibition of the activity of $cPLA_2$ by nobiletin in human keratinocytes

Since arachidonic-acid metabolism can be regulated by the rate-limiting enzyme, $cPLA_2$, as well as by COXs [38], we further examined the effect of nobiletin on the expression and activity of $cPLA_2$ in UVB-irradiated human keratinocytes. As shown in Fig. 3, the release of [^{14}C]arachidonic acid was not influenced for up to 8 h after UVB irradiation. However, nobiletin (64 μM) was found to inhibit the release of [^{14}C]arachidonic acid within 4 h in human keratinocytes that were or were not irradiated with UVB. In addition, we demonstrated that neither nobiletin nor UVB irradiation altered the mRNA levels of $cPLA_2$ in keratinocytes (Fig. 4), indicating that nobiletin might modify the release of arachidonic acid from membrane

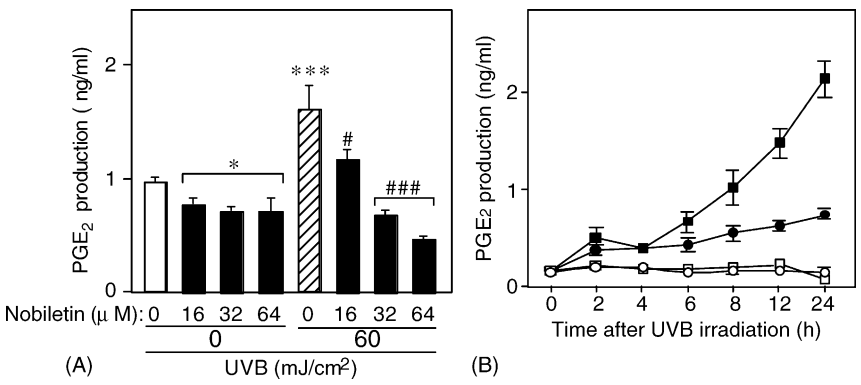

Fig. 1. Inhibition of PGE_2 production by nobiletin in UVB-irradiated human keratinocytes. [A] and [B]: Dose-dependent and time-dependent inhibition of PGE_2 production by nobiletin, respectively. Confluent human keratinocytes at the 3rd passage in 24-well multiplates were pre-treated with nobiletin (16–64 μM) for 30 min and then irradiated with UVB at 60 mJ/cm^2. After the irradiation, the cells were further cultured for 2–24 h and then the harvested culture media were subjected to PGE_2 measurement by radioimmunoassay. The data are indicated as the mean ± S.D. from three individual wells. [A]: (* and ***) Significantly different from untreated cells ($P < 0.05$ and 0.001, respectively); (# and ###) Significantly different from UVB-irradiated cells ($P < 0.05$ and 0.001, respectively). [B]: (●) Untreated cells; (■) UVB-irradiated cells; (○) nobiletin (64 μM)-treated cells; and (□) nobiletin (64 μM) and UVB-treated cells.

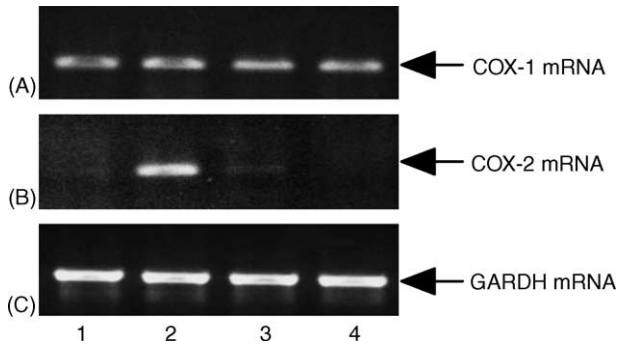

Fig. 2. Nobiletin suppresses the UVB-induced expression of COX-2 mRNA in human keratinocytes. Confluent human keratinocytes at the 4th passage in 60-mm dishes were treated with nobiletin (64 μM) and/or UVB (60 mJ/cm^2) as described in Fig. 1 and then cultured for another 6 h. The isolated RNAs were subjected to semiquantitative RT-PCR for COX-1 [A], COX-2 [B] and GAPDH [C]. Three independent experiments were reproducible and typical data are shown. Lane 1, untreated cells; lane 2, UVB-irradiated cells; lane 3, nobiletin and UVB-treated cells; and lane 4, nobiletin-treated cells.

phospholipids at post-transcriptional levels. Furthermore, when human keratinocytes were treated with nobiletin and a Ca^{2+} ionophore, A23187, which is a potent activator of Ca^{2+}-dependent $cPLA_2$ [40], the A23187-augmented release of [^{14}C]arachidonic acid was completely inhibited by nobiletin (Table 2). Moreover, the Ca^{2+}-dependent $cPLA_2$ activity was similarly abolished by a $cPLA_2$ inhibitor, MAFP. Thus, these results suggest that nobiletin inhibits $cPLA_2$ activity without altering its mRNA expression in human keratinocytes.

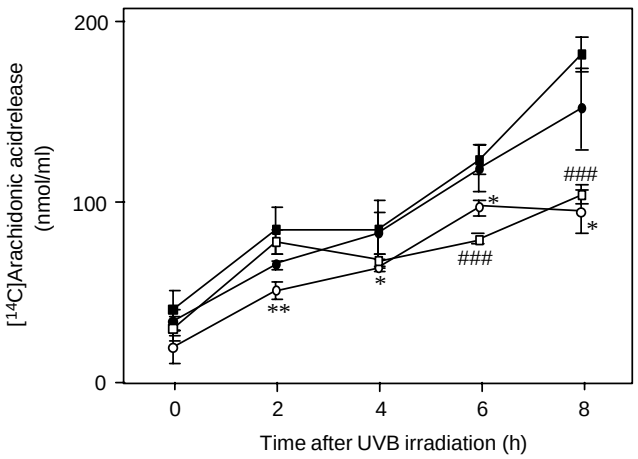

Fig. 3. Nobiletin inhibits the release of [^{14}C]arachidonic acid from phospholipid membranes in human keratinocytes. Phospholipid membranes of human keratinocytes at the 3rd passage in 24-well multiplates were labeled with [^{14}C]arachidonic acid for 24 h and then treated with nobiletin (64 μM) and/or UVB (60 mJ/cm^2) as described in Fig. 1. The radioactivity of [^{14}C]arachidonic acid released into the culture media was measured at indicated periods. The data are indicated as the mean ± S.D. from three individual wells. (●) untreated cells; (■), UVB-irradiated cells; (○) nobiletin-treated cells; and (□) nobiletin and UVB-treated cells. (∗ and ∗∗) Significantly different from untreated cells ($P < 0.05$ and 0.01, respectively). (###) Significantly different from UVB-irradiated cells ($P < 0.001$).

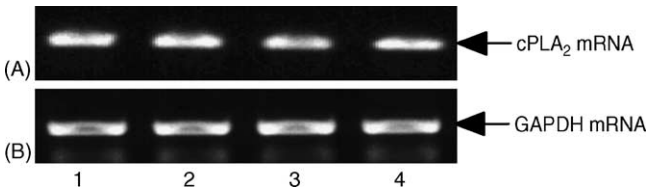

Fig. 4. No alteration of $cPLA_2$ mRNA expression in UVB-irradiated and nobiletin-treated human keratinocytes. Confluent human keratinocytes at the 4th passage in 60-mm dishes were treated with nobiletin (64 μM) and/or UVB (60 mJ/cm^2) as described in Fig. 1 and then cultured for another 8 h. The isolated RNAs were subjected to semiquantitative RT-PCR for $cPLA_2$ [A] and GAPDH [B]. Three independent experiments were reproducible and typical data are shown. Lane1, untreated cells; lane 2, nobiletin-treated cells; lane 3, UVB-irradiated cells and lane 4, nobiletin and UVB-treated cells.

3.4. Inhibition of TEWL and decrease of epidermal thickness by nobiletin in UVB-irradiated mouse skin

Next, we investigated the effects of nobiletin on skin photodamage in hairless mice in vivo. As shown in Table 3, UVB irradiation caused an increase of TEWL (1.6-fold) in skin. However, this augmentation was completely abolished in the nobiletin-treated mice ($P < 0.001$). In

Table 2
Nobiletin inhibits Ca^{2+}-dependent $cPLA_2$ activity in human keratinocytes

Treatments	Release of [^{14}C]arachidonic acid (nmol/ml)	Relative activity (% of A23187 treatment)
None	191.30 ± 8.70	57.4
Nobiletin (64 μM)	178.26 ± 3.48*	53.5
A23187 (1 μM)	333.04 ± 18.26***	100.0
A23187 (1 μM) + Nobiletin (64 μM)	175.65 ± 34.78$^{\#\#\#}$	52.7
A23187 (1 μM) + MAFP (10 μM)	156.52 ± 15.65$^{\#\#\#}$	47.0

[^{14}C]Arachidonic-acid-labeled human keratinocytes at the 3rd passage in 24-well multiplates were treated with A23187 (1 μM), nobiletin (64 μM) and/or MAFP (1 μM) for 8 h and then the radioactivity of [^{14}C]arachidonic acid released into the culture media was measured. The data are indicated as the mean ± S.D. from three individual wells. (∗ and ∗∗∗) Significantly different from untreated cells (none) ($P < 0.05$ and 0.001, respectively). (###) Significantly different from A23187-treated cells ($P < 0.001$).

Table 3
Effects of nobiletin on TEWL and epidermal thickness in UVB-irradiated mouse skin

Treatments	TEWL (g/m^2 h)[a]	Epidermal thickness (μm)[b]
None	12.08 ± 0.83	28.33 ± 3.33
UVB	19.79 ± 3.13**	38.33 ± 2.78*
UVB + nobiletin	12.50 ± 2.50$^{\#}$	33.89 ± 1.00$^{\#}$

(∗ and ∗∗) Significantly different from untreated skin (none) ($P < 0.05$ and 0.01, respectively). (#) Significantly different from UVB-irradiated skin ($P < 0.001$).

[a] Data of TEWL are indicated as the mean ± S.D. from five individual mice.

[b] Data of epidermal thickness in hematoxylin-stained cutaneous tissues are indicated as the mean ± S.D. from 15 individual areas of five mice.

addition, the hyperplasia of the epidermis in UVB-irradiated mice was found to decrease upon nobiletin treatment ($P < 0.01$). Therefore, it is suggested that nobiletin prevents photoaging determined by TEWL and epidermal hyperplasia resulting from UVB irradiation in vivo.

4. Discussion

Acute and chronic UV irradiation causes erythema (sunburn), immunosuppression, and photoaging determined by epidermal thickness and extracellular matrix remodeling of the dermis [1,3,16]. It has been reported that UVB irradiation increases the levels of epidermal PGE_2, which is associated with erythema and the infiltration of inflammatory cells such as mononuclear cells and neutrophils [8–12]. Furthermore, PGE_2 and COX-2 have been reported to participate in the proliferation and differentiation of keratinocytes [41] and as well as in carcinogenesis [42]. Therefore, it is likely that PGE_2 and COX-2 are target molecules in therapies for preventing photoinflammation and sequential skin damage such as photoaging and tumor formation.

Flavonoids from medicinal plants exert various pharmacological efficacies including the prevention and repair of the detrimental effects of UV exposure of the skin [16,25]. Silymarin, a plant flavonoid from milk thistle (*Silybum marianum* L. Gaertn) has been reported to prevent photocarcinogenesis in mice by inhibiting UVB-induced immunosuppression [26]. In addition, nobiletin has been reported to possess novel pharmacological efficacies such as anti-inflammatory, anti-tumor invasive, and chondroprotective activities [27,28,30,31]. In the present study, nobiletin inhibited the UVB-induced production of PGE_2 by the suppression of COX-2 expression in human keratinocytes. In addition, nobiletin was found to depress epidermal thickness and to prevent TEWL in UVB-irradiated hairless mice. Therefore, it is likely that nobiletin acts as an anti-photoinflammatory agent by inhibiting COX-2-dependent PGE_2 production and may be ultimately effective for preventing photoaging in vivo.

The release of arachidonic acid from phospholipids is the step in the biosynthesis of PGs, in which $cPLA_2$ is intrinsically involved [15]. The activity of $cPLA_2$ is regulated by the intracellular Ca^{2+} level, an increase of which stimulates the translocation of $cPLA_2$ to the membrane [43]. In the present study, arachidonic-acid release was inhibited in nobiletin-treated keratinocytes. Furthermore, the inhibition of arachidonic-acid release by nobiletin was observed in human keratinocytes treated with a calcium ionophore, A23187, by which $cPLA_2$ is activated [15,43]. Therefore, in addition to the known inhibitory effect of nobiletin on COX-2 expression, we provide novel evidence that nobiletin inhibits the Ca^{2+}-dependent activation of $cPLA_2$ in human keratinocytes. Moreover, it is likely that the decrease in basal levels of PGE_2 by nobiletin results from the inhibition of $cPLA_2$ activity.

It has been reported that UVB irradiation of the skin induces reactive-oxygen species such as the superoxide anion, the hydroxyl radical, and the peroxy radical, which trigger the onset of skin diseases including inflammation and tumor promotion by a number of mechanisms including DNA damage, lipid peroxidation, and alteration of enzyme activity [4,19,44]. With respect to the photoprotection by plant flavonoids, silymarin inhibits the UVB-induced production of hydrogen peroxide and nitric oxide in mouse skin [26]. Murakami et al. [29] also reported that nobiletin inhibits the generation of superoxide, hydrogen peroxide and nitric oxide in phorbol ester-treated mouse skin. Since UVB-induced oxidative stress augments the epidermal expression of COX-2 [12], the suppression of UVB-induced COX-2 expression by nobiletin may be due to its inhibition of the generation of reactive-oxygen species in human keratinocytes. Moreover, it has been reported that flavonoids such as ginkgetin and quercetin inhibit epidermal $cPLA_2$ activity in guinea pig [45]. However, it remains unclear whether nobiletin directly regulates the activity of $cPLA_2$ in human keratinocytes. Further experiments will be required for addressing the mechanisms of nobiletin's photoprotective actions.

In conclusion, we provide novel evidence that nobiletin inhibits the UVB-induced production of PGE_2 not only by suppressing the expression of COX-2 but also by decreasing the activity of $cPLA_2$ in human keratinocytes. Furthermore, topical treatment with nobiletin is likely to be an efficient therapy for epidermal repairs in UVB-damaged skin. Thus, nobiletin may be useful as a novel sunscreen reagent to be applied for protection against photoinflammation and photoaging.

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References

[1] Hruza LA, Pentland AP. Mechanisms of UV-induced inflammation. J Invest Dermatol 1993;100:35S–41S.
[2] Brach DE. Sunlight and the onset of skin cancer. Trends Genet 1997;13:410–4.
[3] Schwarz T. Photoimmunosuppression. Photodermatol Photoimmunol Photomed 2002;18:141–5.
[4] Miller CC, Hale P, Pentland P. Ultraviolet B injury increases prostaglandin synthesis through a tyrosine kinase-dependent pathway. Evidence for UVB-induced epidermal growth factor receptor activation. J Biol Chem 1994;269:3529–33.
[5] Buckman SY, Gresham A, Hale P, Hruza G, Anast J, Masferrer J, et al. COX-2 expression is induced by UVB exposure in human skin: implications for the development of skin cancer. Carcinogenesis 1998;19:723–9.

[6] Wilgus TA, Ross MS, Parrett ML, Oberyszyn TM. Topical application of a selective cyclooxygenase inhibitor suppresses UVB mediated cutaneous inflammation. Prostaglandins Other Lipid Mediat 2000;62: 367–84.
[7] Wilgus TA, Parrett ML, Ross MS, Tober KL, Robertson FM, Oberyszyn TM. Inhibition of ultraviolet light B-induced cutaneous inflammation by a specific cyclooxygenase-2 inhibitor. Adv Exp Med Biol 2002;507:85–92.
[8] Hla T, Neilson K. Human cyclooxygenase-2 cDNA. Proc Natl Acad Sci USA 1992;89:7384–8.
[9] Grewe M, Trefzer U, Ballhorn A, Gyufko K, Henninger H, Krutmann J. Analysis of the mechanism of ultraviolet (UV) B radiation-induced prostaglandin E_2 synthesis by human epidermoid carcinoma cells. J Invest Dermatol 1993;101:528–31.
[10] Pentland AP. Active oxygen mechanisms of UV inflammation. Adv Exp Med Biol 1994;366:87–97.
[11] Buckman SY, Gresham A, Hale P, Hruza G, Anast J, Masferrer J, et al. COX-2 expression is induced by UVB exposure in human skin: implications for the development of skin cancer. Carcinogenesis 1998;19:723–9.
[12] Isoherranen K, Punnonen K, Jansen C, Uotila P. Ultraviolet irradiation induces cyclooxygenase-2 expression in keratinocytes. Br J Dermatol 1999;140:1017–22.
[13] Anderson RR, Parrish JA. The optics of human skin. J Invest Dermatol 1981;77:13–9.
[14] Gresham A, Masferrer J, Chen X, Leal-Khouri S, Pentland AP. Increased synthesis of high-molecular weight $cPLA_2$ mediates early UV-induced PGE_2 in human skin. Am J Physiol 1996;270: C1037–50.
[15] Clark JD, Milona N, Knopf JL. Purification of a 110-kilodalton cytosolic phospholipase A_2 from the human monocytic cell line U937. Proc Natl Acad Sci USA 1990;87:7708–12.
[16] Berneburg M, Plettenberg H, Krutmann J. Photoaging of human skin. Photodermatol Photoimmunol Photomed 2000;16:239–44.
[17] Bykov VJ, Marcusson JA, Hemminki K. Ultraviolet B-induced DNA damage in human skin and its modulation by a sunscreen. Cancer Res 1998;58:2961–4.
[18] Nola I, Kotrulja L. Skin photodamage and lifetime photoprotection. Acta Dermatovenerol Croat 2003;11:32–40.
[19] Pinnell SR. Cutaneous photodamage, oxidative stress, and topical antioxidant protection. J Am Acad Dermatol 2003;48:1–19.
[20] Bissett DL, Chatterjee R, Hannon DP. Photoprotective effect of superoxide-scavenging antioxidants against ultraviolet radiation-induced chronic skin damage in the hairless mouse. Photodermatol Photoimmunol Photomed 1990;7:56–62.
[21] Simics E, Mahunka M, Horkay I, Bohnert E, Moll I, Jung EG. Effect of pentoxifylline on sunburn cell formation in a novel supravital human skin model. Photodermatol Photoimmunol Photomed 2000;16: 278–80.
[22] Lowitt MH. Pentoxifylline attenuates UVB-induced cutaneous erythema. Dermatology 2001;202:44–5.
[23] Harborne JB, Williams CA. Advances in flavonoid research since 1992. Phytochemistry 2000;55:481–504.
[24] Rice-Evans C. Flavonoid antioxidants. Curr Med Chem 2001;8: 797–807.
[25] Singh RP, Agarwal R. Flavonoid antioxidant silymarin and skin cancer. Antioxid Redox Signal 2002;4:655–63.
[26] Katiyar SK. Treatment of silymarin, a plant flavonoid, prevents ultraviolet light-induced immune suppression and oxidative stress in mouse skin. Int J Oncol 2002;21:1213–22.
[27] Ishiwa J, Sato T, Mimaki Y, Sashida Y, Yano M, Ito A. Citrus flavonoid, nobiletin, suppresses the production and gene expression of matrix metalloproteinase 9/gelatinase B in rabbit synovial fibroblasts. J Rheumatol 2000;271:20–5.
[28] Lin N, Sato T, Takayama Y, Mimaki Y, Sashida Y, Yano M, et al. Novel anti-inflammatory actions of nobiletin, a citrus polymethoxy flavonoid, on human synovial fibroblasts and mouse macrophages. Biochem Pharmacol 2003;65:2065–71.
[29] Murakami A, Nakamura Y, Torikai K, Tanaka T, Koshiba T, Koshimizu K, et al. Inhibitory effect of citrus nobiletin on phorbol ester-induced skin inflammation, oxidative stress, and tumor promotion in mice. Cancer Res 2000;60:5059–66.
[30] Minagawa A, Otani Y, Kubota T, Wada N, Furukawa T, Kumai K, et al. The citrus flavonoid, nobiletin, inhibits peritoneal dissemination of human gastric carcinoma in SCID mice. Jpn J Cancer Res 2001; 92:1322–8.
[31] Sato T, Koike L, Miyata Y, Hirata M, Mimaki Y, Sashida Y, et al. Inhibition of activated protein-1 binding activity and phosphatidylinositol 3-kinase pathway by nobiletin, a polymethoxy flavonoid, results in augmentation of tissue inhibitor of metalloproteinases-1 production and suppression of production of matrix metalloproteinase 1 and 9 in human fibrosarcoma HT-1080 cells. Cancer Res 2002;62:1025–9.
[32] Sato T, Kirimura Y, Mori Y. The co-culture of dermal fibroblasts with human epidermal keratinocytes induces increased prostaglandin E_2 production and cyclooxygenase 2 activity in fibroblasts. J Invest Dermatol 1997;109:334–9.
[33] Roth GJ, Stanford N, Majerus PW. Acetylation of prostaglandin synthase by aspirin. Proc Natl Acad Sci USA 1975;72:3073–6.
[34] Futaki N, Takahashi S, Yokoyama M, Arai I, Higuchi S, Otomo S. NS-398, a new anti-inflammatory agent, selectively inhibits prostaglandin G/H synthase/cyclooxygenase (COX-2) activity in vitro. Prostaglandins 1994;47:55–9.
[35] Sato T, Nakajima H, Fujio K, Mori Y. Enhancement of prostaglandin E_2 production by epidermal growth factor requires the coordinate activation of cytosolic phospholipase A_2 and cyclooxygenase 2 in human squamous carcinoma A431 cells. Prostaglandins 1997;53: 355–69.
[36] Ahmed SA, Gogal RM, Walsh JE. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [^{3}H] thymidine incorporation assay. J Immunol Methods 1994;170:211–24.
[37] Funk CD, Funk LB, Kennedy ME, Pong AS, Fitzgerald GA. Human platelet/erythroleukemia cell prostaglandin G/H synthase: cDNA cloning, expression, and gene chromosomal assignment. FASEB J 1991;5:2304–12.
[38] Sharp JD, White DL, Chiou XG, Goodson T, Gamboa GC, McClure D, et al. Molecular cloning and expression of human Ca^{2+}-sensitive cytosolic phospholipase A_2. J Biol Chem 1991;266:14850–3.
[39] Tokunaga K, Nakamura Y, Sakata K, Fujimori K, Ohkubo M, Sawada K, et al. Enhanced expression of a glyceraldehyde-3-phosphate dehydrogenase gene in human lung cancers. Cancer Res 1987;47:5616–9.
[40] Schievella AR, Reiger MK, Smith WL, Lin LL. Calcium-mediated translocation of cytosolic phospholipase A_2 to the nuclear envelope and endoplasmic reticulum. J Biol Chem 1995;270:30749–54.
[41] Pentland AP, Needleman P. Modulation of keratinocyte proliferation in vitro by endogenous prostaglandin synthesis. J Clin Invest 1986;77:246–51.
[42] Dempke W, Rie C, Grothey A, Schmoll HJ. Cyclooxygenase-2: a novel target for cancer chemotherapy? J Cancer Res Clin Oncol 2001; 127:411–7.
[43] Schalkwijk CG, Spaargaren M, Defize LH, Verkleij AJ, van den Bosch H, Boonstra J. Epidermal growth factor (EGF) induces serine phosphorylation-dependent activation and calcium-dependent translocation of the cytosolic phospholipase A_2. Eur J Biochem 1995;231: 593–601.
[44] Goldberg HJ, Viegas MM, Margolis BL, Schlessinger J, Skorecki KL. The tyrosine kinase activity of the epidermal-growth-factor receptor is necessary for phosphlipase A_2 activation. Biochem J 1990;267:461–5.
[45] Kim HR, Pham HT, Ziboh VA. Flavonoids differentially inhibit guinea pig epidermal cytosolic phospholipase A_2. Prostaglandins Leukot Essent Fatty Acids 2001;65:281–6.