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Essential role of Nrf2 in keratinocyte protection from UVA by quercetin

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ABSTRACT

Much of the cell injury caused by ultraviolet A (UVA) irradiation is associated with oxidative stress. Quercetin is a major natural polyphenol that is known to protect cells from UVA-induced damage. Here, we investigated the molecular mechanism of this protection. Quercetin pretreatment strongly suppressed UVA-induced apoptosis in human keratinocyte HaCaT cells, markedly increased protein levels of the transcription factor Nrf2, induced the expression of antioxidative genes, and dramatically reduced the production of reactive oxygen species following UVA irradiation. Importantly, these beneficial effects were greatly attenuated by downregulating Nrf2 expression. Thus, quercetin protects cells from UVA damage mainly by elevating intracellular antioxidative activity via the enhanced accumulation of a transcription factor for antioxidant genes, Nrf2.

Introduction

The major environmental cause of skin damage is excessive UV irradiation, which acutely causes reactions like erythema and sunburn, and over the long-term can result in premature skin ageing and carcinoma [1–6]. Longwave UV radiation (UVA) (320–400 nm) penetrates deeper into the skin than the shorter-wavelength UVB radiation, and affects the deep dermis. Exposure of the skin to UVA increases the cellular levels of reactive oxygen species (ROS), which has been linked to apoptosis and to the damage of lipids, proteins, and nucleic acids [7].

Nrf2 is a well-characterized transcription factor that plays an important role in the antioxidant response element (ARE)-mediated expression of a group of genes encoding phase II detoxification enzymes and antioxidant proteins, such as hemeoxygenase 1 (HO-1), peroxiredoxin 1 (Prx I), NAD(P)H quinone oxidoreductase 1 (NQO1), and glutamate-cysteine ligase [8,9]. These enzymes are crucial for protecting cells from electrophile toxicity, oxidative stress, and to prevent carcinogenesis. Under basal conditions, Nrf2 is negatively regulated by Kelch-like ECH-associated protein 1 (Keap1), which facilitates the degradation of Nrf2 through the proteasome [10]. Electrophilic compounds are believed to attack the reactive cysteine residues in the Keap1 intervening region (IVR), leading to a conformational change in the Keap1–Nrf2 association motif and thereby the dissociation of Nrf2 from Keap1 [11–13]. Nrf2 plays an important role in protecting skin against UVA irradiation [14]. One report showed that the plant-derived polyphenol quercetin enhances Nrf2-dependent ARE activity, induces *ho-1* expression, and protects hepatocytes from ethanol-derived oxidative stress [15].

One way to prevent UVA-induced skin damage is to apply antioxidants to the skin. Quercetin is a major polyphenol contained in fruits and vegetables such as citrus, onions, kale, and broccoli. In vitro and in vivo, it protects animal cells against the pathogeneses associated with oxidative stress, including carcinogenesis, inflammation, and viral infection [16–20]. Quercetin has been proposed to exert these beneficial effects by scavenging reactive oxygen species or chelating metal cations [21–26].

Quercetin enhances ARE-binding activity and Nrf2-mediated transcription activity [27], and it prevents UVA damage in rats [21]. However, the role of Nrf2 in quercetin's protective role against UVA-induced damage remains intriguing but unclear. In this study, we examined the mechanisms of quercetin's protection of human keratinocyte-derived HaCaT cells from UVA-induced damage. We demonstrate the molecular mechanism of quercetin's antioxidative effect and the importance of Nrf2 activation in protecting cells.

Materials and methods

Cell culture and quercetin treatment. Human keratinocytes of the HaCaT cell line were grown in Dulbecco's modified Eagle's medium (DMEM) containing streptomycin (100 U/ml), penicillin (0.1 mg/ml), and heat-inactivated 10% FBS. The cells were cultured in a

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humidified atmosphere with 5% CO_2 at 37 °C. Quercetin (Nacalai tesque, Japan) was dissolved in DMSO and added to the culture medium as indicated.

UVA irradiation. Before UVA irradiation, cells pretreated with quercetin for 12 h and untreated control cells were washed with PBS, and the medium was replaced with phenol-red-free DMEM containing 5% FBS and 30 mM HEPES. Then, the cells were irradiated with UVA using FL20SBLB lamps (Toshiba, Japan) with a peak emission frequency of 352 nm. Wavelengths below 320 nm were blocked by an ATG filter UV-35 (Asahi Technoglass, Japan).

Western blotting analysis. Whole-cell extracts were obtained using RIPA buffer. The protein concentrations were determined by the BCA protein assay (Pierce, IL). Western blot analysis was performed as described previously [28].

Plasmid DNA transfection and luciferase activity. Cells at 50% confluency were transfected with plasmids using Fugene HD reagent (Roche, Switzerland). Briefly, a transfection complex containing 1 μg of ARE-luciferase plasmid DNA [29], 0.02 μg of pRLtk plasmid (Promega, WI), and 3 μl of Fugene HD in 500 μl of opti-MEM was added to the cells. The cells were incubated for 16 h, and quercetin was then added to the medium. After another 12 h, the cells were lysed, and the Renilla and firefly luciferase activities were measured using a Dual-luciferase assay kit (Promega). The luciferase activity was normalized to the Renilla luciferase activity.

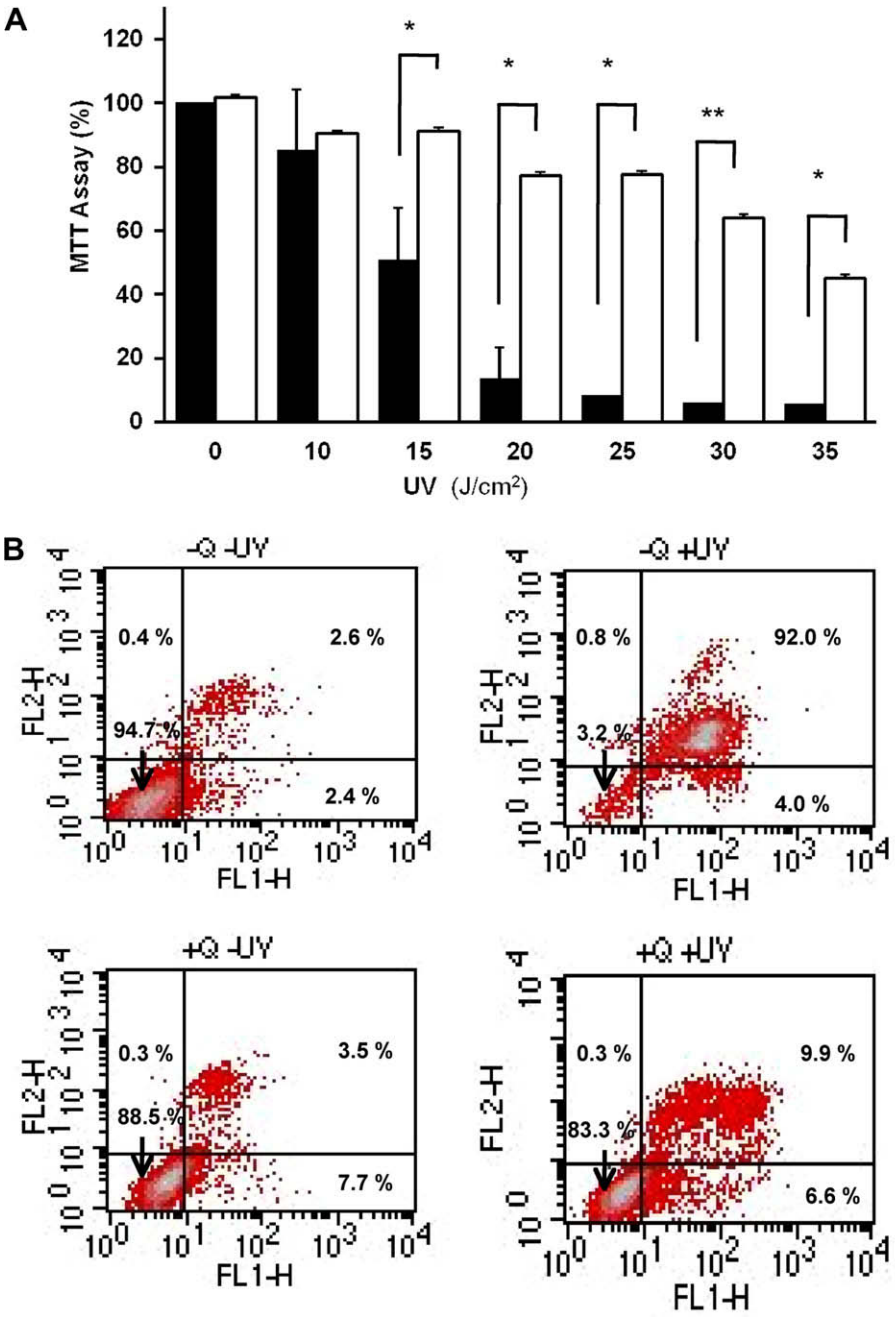

Fig. 1. Quercetin treatment protects HaCaT cells from UVA-induced apoptosis. (A) HaCaT cells were treated with 50 μM quercetin for 12 h (open columns) or were not treated (filled columns), and were then irradiated with various UVA doses. (B) The distributions of living and dead cells were determined by FACS. For this experiment, the control HaCaT cells and the pretreated cells (50 μM quercetin for 12 h) were irradiated with 30 J/cm^2 UVA.

MTT assay. To assay cell viability, a tetrazolium salt, 3-(4,5-dimethylthiazol-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml), was added to the culture medium of HaCaT cells and incubated for 3 h (37 °C). The medium was removed, and the cells were lysed with 1 ml of dimethyl sulfoxide. The absorbance was measured at 540 nm.

Detection of ROS generation and mitochondrial damage. Intracellular ROS generation was measured using 2′,7′-dichlorofluorescein diacetate (DCFH-DA). HaCaT cells pretreated with quercetin for 12 h and untreated cells were subjected to UVA irradiation. Then, DCFH-DA (20 μM) and MitoTracker Deep Red (150 nM) were added to the culture medium and the cells were incubated for 30 min. The cells were then washed five times with culture medium, and fluorescent images were acquired with a confocal laser scanning microscope, TCS SP2 (Leica, Microsystems, Germany).

Intracellular glutathione levels. The cells were washed three times with PBS, and the cellular glutathione was extracted with 5% trichloroacetic acid. The intracellular glutathione content was measured by an enzymatic method based on catalytic action [30].

Flow cytometry. Analysis of apoptosis was performed using an annexin-V/PI staining kit (Roche). Briefly, cells were irradiated with UVA (30 J/cm^2), washed with PBS, collected by trypsinization, and stained with annexin-V and PI (propidium iodide) for 15 min. Flow cytometry was performed using a FACS Calibur (Becton–Dickinson, NJ).

Quantitative real-time PCR. Quantitative real-time PCR was carried out as described previously [28]. The expression levels were normalized to that of *gapdh* mRNA.

siRNA transfection. The siRNA target sequence for Nrf2 was 5′-AAG AGT ATG AGC TGG AAA AAC-3′. siRNA (80 nM) was transfected with Lipofectamine RNAiMax (Invitrogen, CA), according to the manufacturer's instructions.

Statistical analysis. All experiments were repeated at least three times, and the results are expressed as the mean ± SD. Student's *t*-test was used to analyze statistical significance. The significance limits were set at $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

Results

Quercetin protects HaCaT cells from UVA-induced apoptosis

The cytotoxity of UVA was determined by the MTT assay (Fig. 1A). In control cells, 10 J/cm^2 irradiation decreased the HaCaT cell viability, and nearly 90% of the cells were killed by 20 J/cm^2 UVA. However, quercetin-treated cells were much more resistant: roughly 50% survived even after 35 J/cm^2 irradiation. The distribution of living and dead cells was determined by flow cytometry, and the difference between the control and quercetin-treated cells is shown in Fig. 1B. After UVA irradiation, 96% of the total control cells were apoptotic cells (annexin-positive/PI-negative or annexin-positive/PI-positive cells), while only 16.5% of the quercetin-treated cells were apoptotic cells, indicating quercetin's marked protective effect against UVA-induced apoptosis.

Quercetin suppresses ROS production, morphological changes in mitochondria, and the reduction of intracellular GSH

HaCaT cells were treated with DCFH-DA to visualize the ROS production from UVA irradiation. As shown in Fig. 2A, strong signals derived from excited DCFH-DA were detected in control cells

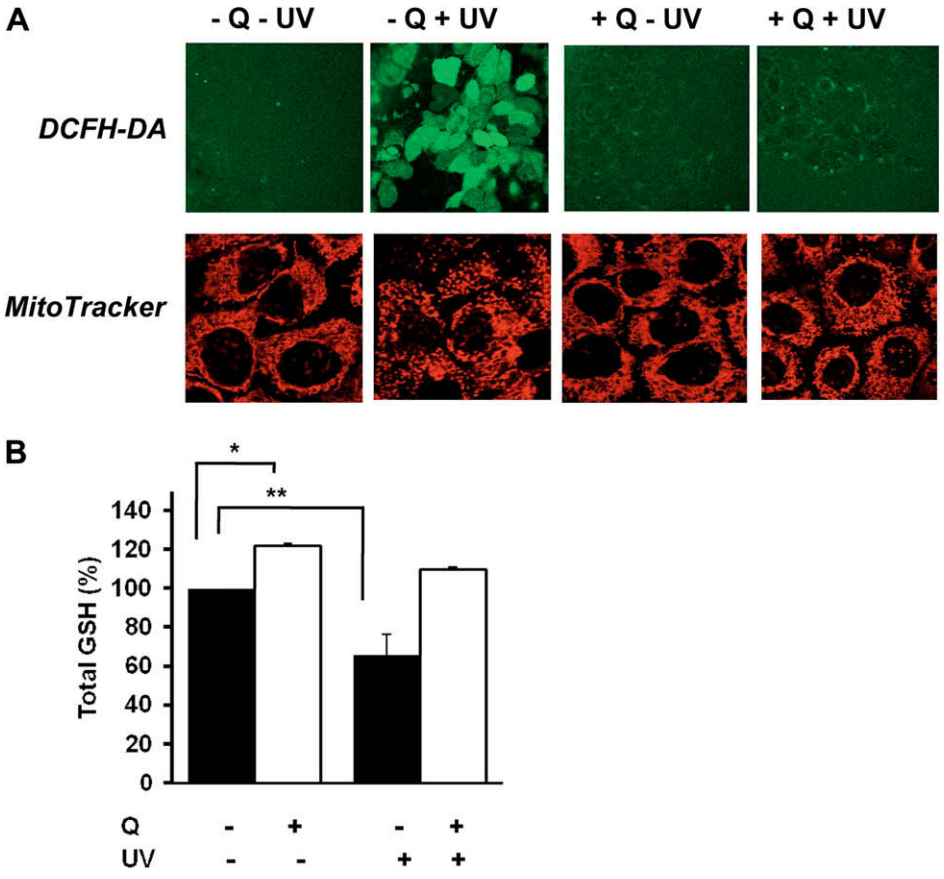

Fig. 2. Modulation of UVA-induced ROS production and oxidative damage by quercetin. (A) HaCaT cells were pretreated with 50 μM quercetin for 12 h (control cells were not treated) and irradiated with 15 J/cm^2 UVA. The signals were imaged with confocal laser scanning microscopy. (magnification; DCFH-DA, ×200; Mito Tracker, ×630) (B) Changes in intracellular GSH level after UVA (15 J/cm^2) irradiation with or without quercetin treatment.

(−Q, +UV) when the cells were exposed to 15 J/cm^2 UVA. Notably, the strong signals were located in the mitochondria. In contrast, the DCFH-DA signals were marginally detectable in UVA-irradiated, quercetin-treated cells (+Q, +UV). These results clearly suggest that quercetin suppresses UVA-mediated ROS production. In addition, morphological changes in the mitochondria were observed in the UVA-irradiated control cells. However, the quercetin-treated cells did not exhibit this response (Fig. 2A). To evaluate the extent of oxidative damage, the intracellular glutathione content was measured. UVA irradiation markedly reduced the total GSH in the control cells, but there was no significant decrease in the quercetin-treated cells (Fig. 2B), indicating that quercetin has a protective role in maintaining the cellular GSH content.

Quercetin upregulates Nrf2 and induces the expression of ARE-driven genes

To investigate the molecular mechanism of quercetin's antioxidative effect, we examined its Nrf2-activating capacity. Quercetin treatment (1–100 μM) dose-dependently caused the accumulation of Nrf2 protein (Fig. 3A) without impairing the cell viability (data not shown). Next, we investigated the effects of transcriptional activation by Nrf2 in HaCaT cells, by transfecting them with ARE-luciferase reporter plasmids. The luciferase activity was increased approximately 10-fold after quercetin treatment (Fig. 3B), indicating that quercetin treatment activated ARE-regulated gene expression. Quantitative real-time PCR analysis revealed that quercetin treatment upregulated the *ho-1* and *gclm* mRNAs, Nrf2 target genes (Fig. 3C).

Nrf2 knockdown diminishes the protective effect of quercetin

To demonstrate the importance of Nrf2 upregulation, we developed an *Nrf2* gene knockdown model in HaCaT cells by using siRNA transfection. The effect of knocking down the gene for Nrf2 was confirmed by Western blotting analysis, which showed a significant decrease in Nrf2 protein levels following the siRNA transfection (Fig. 4A). The Nrf2 knockdown was effective even with a 12-h quercetin treatment. That is, the transfection with Nrf2-siRNA almost completely abrogated the protective effect of quercetin; HaCaT cells did not survive 30 J/cm^2 UVA irradiation even in the presence of quercetin (Fig. 4B).

Discussion

In the present study, we showed that one of the major polyphenols, quercetin, markedly protects HaCaT cells from UVA-induced apoptosis. Quercetin causes Nrf2 accumulation and promotes ARE-driven transcriptional activity, while inhibiting UVA-induced ROS production. Notably, quercetin's protection was almost completely blocked by knocking down Nrf2 expression.

UVA is toxic because it generates ROS in cells and induces apoptosis. The transcription factor Nrf2 is a known key regulatory factor in antioxidative stress protein expression. The molecular mechanism of quercetin's activation of Nrf2 is not yet clear, since quercetin itself is not an electrophile. Quercetin induces ARE-driven transcription activity via the upregulation of Nrf2 mRNA and by stabilizing Nrf2 [27]. It reduces the level of the Keap1 protein at the post-transcriptional level through the proteasome pathway, resulting in a high ratio of Nrf2/Keap1 [27]. Nakazato et al. have reported that a different polyphenol, (−)-epigallocatechin-3-gallate, induces ROS production in cells [31], which suggests that polyphenols may have a potent ability to modify the SH-residue in Keap1 through ROS production. However, we did not detect ROS signals from DCFH-DA in HaCaT cells during quercetin treatment (Fig. 2A). This result implies that an ROS-independent pathway is involved in the Nrf2 activation by quercetin.

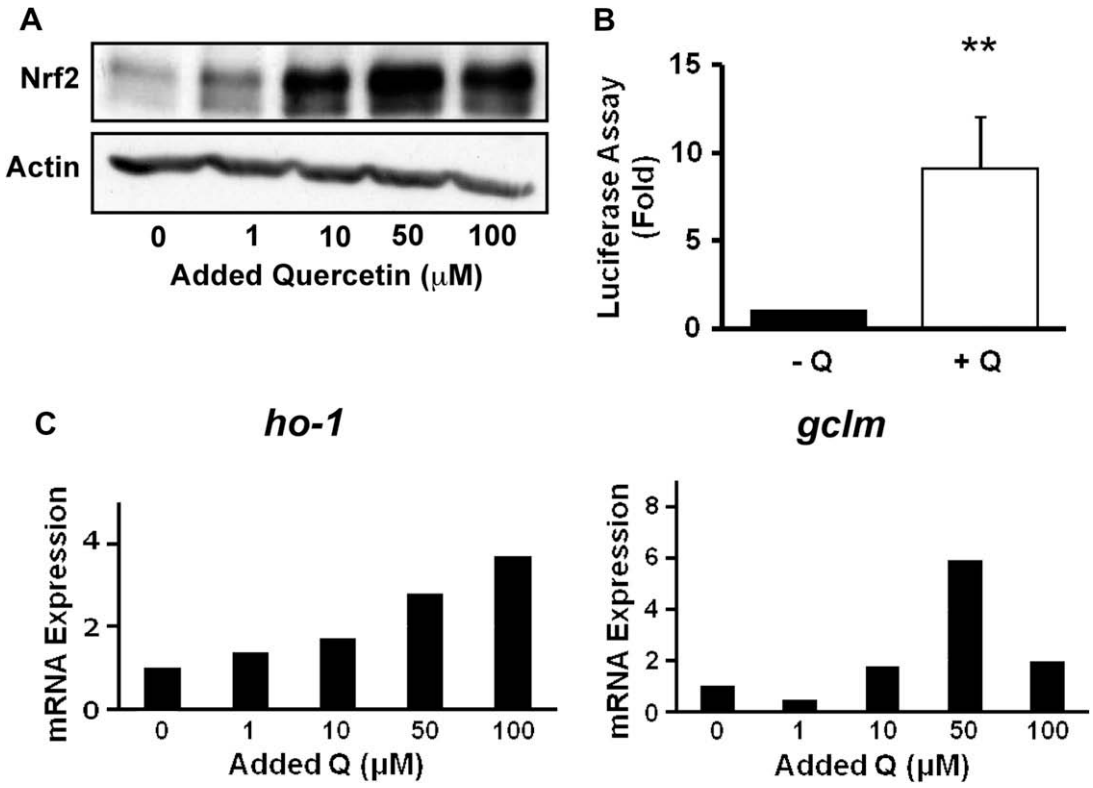

Fig. 3. Upregulation of Nrf2 and the expression of Nrf2 target genes induced by quercetin. (A) Western blotting of total cell lysate (40 μg of protein per lane). (B) Luciferase activity in HaCaT cells transfected with the ARE-Luciferase reporter plasmid and treated with quercetin for 12 h (open columns) and in transfected untreated control cells (filled columns). (C) The mRNA expression levels of Nrf2 target genes *ho-1* and *gclm* were analyzed by quantitative real-time PCR. The expression levels were normalized to the level of *gapdh* mRNA.

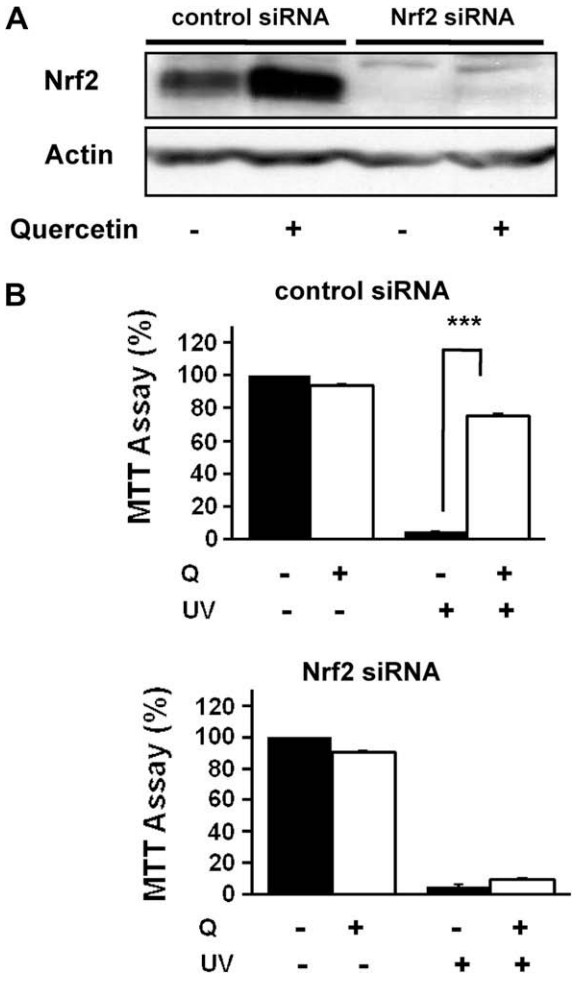

Fig. 4. Nrf2 knockdown attenuates the protective effect of quercetin. (A) Knockdown evaluation by Western blot. HaCaT cells were transfected with a specific siRNA against Nrf2 or a non-silencing control. After 48 h of transfection, the cells were incubated with or without quercetin for 12 h. (B) Control and Nrf2 siRNA-treated HaCaT cells were incubated with (open columns) or without (filled columns) quercetin, and were irradiated with 30 J/cm^2 UVA. The cell viabilities were determined by the MTT assay.

Besides quercetin, we examined the effect on Nrf2 accumulation of other polyphenols: resveratrol, caffeic acid, epicatechin, epigallocatechin, epigallocatechin gallate, catechin, baicalein, and kaempferol. All of these polyphenols, except epicatechin and epigallocatechin, also upregulated Nrf2. Resveratrol and kaempferol, in particular, had strong effects on Nrf2 accumulation (data not shown). Interestingly, these two compounds have the same chemical backbone, 1,3-dihydroxybenzene, suggesting a possible structure–activity relationship between the chemical backbone of polyphenols and the degree of Nrf2 activation. The precise molecular mechanism underlying Nrf2 activation by polyphenols needs further investigation.

In summary, our study has provided the molecular mechanism by which quercetin protects cells from UVA irradiation. Since the activation of the antioxidant system via Nrf2 is critical for the protection of skin cells from UVA, Nrf2-activating polyphenols like quercetin may be a desirable supplement in skin care preparations.

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