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Chrysin Protects Epidermal Keratinocytes from UVA- and UVB-Induced Damage

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ABSTRACT: Chrysin (5,7-dihydroxyflavone), a natural flavonoid occurring in various plants and foods such as propolis and honey, reportedly opposes inflammation and carcinogenesis, but has rarely been applied in skin care. This study, therefore, aimed to explore the roles of chrysin in protection against UV-induced damage in HaCaT keratinocytes. Results showed that chrysin can attenuate apoptosis, reactive oxygen species (ROS) production, and cyclooxygenase 2 (COX-2) expression induced by UVB and UVA. Chrysin predominantly reversed the down-regulation of aquaporin 3 (AQP-3) by UVB. It predominantly reversed JNK activation and also mildly inhibited p38 activation triggered by UVA and UVB. Animal studies revealed that chrysin's topical application demonstrated efficient percutaneous absorption and no skin irritation. Overall, results demonstrated significant benefits of chrysin on the protection of keratinocytes against UVA- and UVB-induced injuries and suggested its potential use in skin photoprotection.

KEYWORDS: UV, keratinocyte, chrysin, apoptosis, COX-2, aquaporin-3

INTRODUCTION

Solar ultraviolet (UV) light can be divided into three wavelength regions including UVC (200−280 nm), UVB (280−320 nm), and UVA (320−400 nm). UVB and UVA can both penetrate the atmosphere and cause the most known skin disorders.[1] Acute UV irradiation can elicit various responses including sunburn, inflammation, DNA damage, and apoptosis.[2−4] Chronic and repetitive UV irradiation can lead to photoaging, sustained immune suppression, and carcinogenesis of the skin.[2,5] Multiple lines of evidence have demonstrated that UV light is the major environmental carcinogenic factor in the development of nonmelanoma skin cancers, such as squamous cell carcinoma (SCC) and basal cell carcinoma (BCC). UV irradiation alone can be a competent carcinogen responsible for the three stages of tumorigenesis, including initiation, promotion, and progression.[6]

Both UVB and UVA can induce the apoptotic program. The mechanisms of UV-induced apoptosis involve synergistic contributions from three independent pathways, including DNA damage, death receptor activation, and formation of reactive oxygen species (ROS).[6] The production of ROS, triggered by UVA and UVB, directly or indirectly affects the signaling pathways of cell death and survival and also mediates various UV-induced cutaneous reactions, including inflammation, photosensitivity, and carcinogenic processes.[7] Several signaling pathways, such as mitogen-activated protein kinases (MAPKs), phosphoinositide 3′-kinase (PI3K)/Akt, p53, activator protein-1 (AP-1), and nuclear factor-κ B (NF-κB) also have critical involvement in the regulation of cell death triggered by UV.[1,6,7]

Cyclo-oxygenase-2 (COX-2) is usually expressed in perturbed epithelial tissues, triggered by multiple mitogenic and inflammatory stimuli, including growth factors and UV light.[4] Prostaglandins (PGs) produced by COX-2 play critical roles in various pathophysiological processes, such as inflammation, reproduction, nociception, and gastrointestinal protection.[4,8] Previous research has established the role of COX-2 in skin cancer development. In one investigation, overexpression of COX-2 in human BCC cell lines resulted in significant resistance to UV-induced apoptosis.[9] In animal studies, mice deficient in *COX-2* showed dramatically decreased UV-induced skin tumorigenesis, whereas overexpression of *COX-2* in transgenic mice led to enhanced tumorigenesis.[4] Inhibition of COX-2 diminished cutaneous inflammation and reduced UV-induced skin tumor formation in two separate studies in mice.[8,10]

UV irradiation can also cause human skin dehydration, which might be attributed to UV-induced down-regulation of aquaporin 3 (AQP-3).[11] AQPs are a family of small hydrophobic integral membrane proteins that function as water selective pores and facilitate osmotically driven water transport.[12,13] Basal epidermal keratinocytes express AQP-3 in their plasma membranes. Experimental findings in AQP-3-knockout mice illustrate the involvement of AQP-3 in water and glycerol transportation, which contributes to skin hydration, barrier recovery, wound healing, and tumorigenesis.[12] Reducing the suppression of AQP-3 by UV light might, therefore, be beneficial in photoprotection.

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Flavonoids are polyphenolic compounds and benzo-γ-pyrone derivatives. They are naturally occurring components in a variety of daily food items and have a range of biological functions.[14,15] Many studies have indicated that the flavonoids can suppress carcinogenesis through multiple mechanisms, including inhibition of the stages of initiation, promotion, and progression.[15] Honey, which contains various flavonoids, has reported anticancer properties and can induce apoptosis and inhibit metastasis.[14,16,17] Chrysin (5,7-dihydroxyflavone) is a natural flavonoid, extracted from honey, propolis, and various plants.[16,18] Recent studies have revealed the pleiotropic biological activities of chrysin, such as antioxidation, anti-inflammation, anticancer, and inactivation of infectious agents.[19−21] However, other novel functions and mechanisms of action of chrysin in skin biology remain elusive.

Use of botanical agents to decrease the occurrence of skin cancer by photochemoprevention has increasingly gained attention. The present group's previous studies showed the photoprotective effects of various flavonoids, such as (−)-epicatechin-3-gallate and myricetin, on keratinocytes.[22,23] UV-induced skin damage or cancer formation can be slow, insidious processes; therefore, photochemoprevention is mandatory in daily life to prevent severe damage. Consequently, this study aimed to explore the protective effects of chrysin, an easily accessible natural flavonoid, on UV-induced damage in keratinocytes and to elucidate the underlying signaling pathways. We also evaluated the percutaneous absorption and safety after topical application of chrysin on mice models to determine the feasibility of clinical use.

MATERIALS AND METHODS

Reagents. Chrysin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), aprotinin, leupeptin, phenylmethanesulfonyl fluoride (PMSF), sodium fluoride (NaF), and sodium orthovanadate were purchased from Sigma Chemical Co. (St. Louis, MO). Antibodies raised against phosphoextracellular signal-regulated kinase 1/2 (ERK1/2) and AQP-3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). ERK1/2, c-Jun N-terminal kinase (JNK), and phospho-p38 antibodies were obtained from R&D Systems, Inc. (Minneapolis, MN). Antibodies raised against p38, p-JNK, and actin were from Cell Signaling Technology (Beverly, MA), and the COX-2 antibody was from BD Biosciences (Fullerton, CA).

Cell Cultures. Human immortalized keratinocytes (HaCaT cells) were a gift from Dr. Yih-Jing Lee of Fu Jen Catholic University and maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (GibcoBRL, Invitrogen Life Technologies, Carlsbad, CA), 100 units/mL penicillin, and 100 μg/mL streptomycin (Sigma).

UV Irradiation. HaCaT cells were preincubated with chrysin in serum-free DMEM for 24 h. After two washings, the cells were incubated with phosphate-buffered saline (PBS). UV irradiation was performed immediately as suggested by the manufacturer. Briefly, cells were irradiated in a Bio-Sun system illuminator (Vilber Lourmat, France). UVB was supplied by a closely spaced array of two UV lamps with a UV peak of 312 nm. UVA was supplied by a closely spaced array of four UV lamps with a UV peak of 365 nm. Both kinds of UV lamps delivered uniform irradiation at a distance of 10 cm. After UV exposure, cells were fed fresh serum-free DMEM. The control groups without treatment of chrysin were processed in the same way, except for UV irradiation.

Cell Viability Assay. The viability of cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously.[24]

Cell Lysate Preparation and Western Blot Analysis. Cells were collected and washed after indicated treatments. Total protein was separated using electrophoresis on SDS−10% polyacrylamide gels. The proteins were then electroblotted onto PVDF membranes and probed using antibodies. The general procedures were as described previously.[24]

Cell Cycle Analysis by Flow Cytometry. The cell cycle was determined using a Partec CyFlow ML flow cytometer (Partech, Munster, Germany). At indicated time points, cells were harvested by trypsinization, washed twice with PBS, resuspended in PBS, and then fixed with ice-cold methanol. Thereafter, Triton X-100 was used for permeation, and propidium iodide solution was added and incubated. The samples were analyzed by flow cytometry to define the sub G1, G0/1, S, and G2 + M phases. The sub G1 phase was quantified to analyze the nonviable cell population.

Intracellular ROS Detection. Intracellular production of ROS was detected as described previously.[22] Briefly, HaCaT keratinocytes starved in DMEM were pretreated with various concentrations of chrysin for 24 h. Cells were washed with PBS and then treated with dihydrorhodamine (DHR) 123 (10 μg/mL) in DMEM for 30 min. After washing, cells were irradiated with UV and then collected and centrifuged. The cells were analyzed immediately using a flow cytometer (Partech), at excitation and emission wavelengths of 488 and 525 nm, respectively. Fluorescence signals were collected to calculate the mean fluorescence intensities from single cells.

Animal Experiments and Skin Physiology Studies. *Animals.* Eight-week-old female ICR-Foxn/^{nu} mice were obtained from the National Laboratory Animal Center, Taipei, Taiwan. Animals were housed and handled according to institutional guidelines. Briefly, mice were housed eight per cage with controlled temperature (21−25 °C), humidity (60 ± 5%), and light (12/12 h light/dark cycle) for 1 week. Alfalfa-free food (5058, LabDiet, Framingham, MA) and water were given ad libitum. All of the animal experimental protocols were reviewed by the committee and conducted after obtaining the Affidavit of Approval of Animal Use Protocol of Fu Jen Catholic University.

In Vitro Skin Permeation. In vitro skin permeation experiments were performed using a Franz diffusion cell.[25] Cellulose membrane (molecular porous membrane, Spectral/Por 3 membrane, MW cutoff 3500, Montluçon Cedex, France), the abdominal skin of nude mice stripped by tape 20 times, and the intact abdominal skin of nude mice were mounted on the receptor compartment with the SC-side facing upward into the donor compartment. The donor medium was the vehicle containing chrysin. The receptor was 10 mL of pH 7.4 phosphate buffer. The stirring rate and temperature were maintained at 600 rpm and 37 °C, respectively. At 2, 4, 6, 8, 10, 12, 24, 30, 36, and 48 h after application, a 300 μL aliquot of the receptor medium was withdrawn and immediately replaced with an equal volume of fresh buffer. The amounts of drugs were determined using high-performance liquid chromatography (HPLC) . The amount of chrysin retained in the skin was examined at the end of the in vitro permeation experiment. After washing, the skin sample was weighed, cut with scissors, positioned in a glass homogenizer containing 1 mL of methanol, and ground for 5 min using an electric stirrer. The resulting solution was centrifuged for 10 min at 10000 rpm. The supernatant was analyzed using HPLC to determine the chrysin content.

In Vivo Topical Application. Chrysin in PEG400/ethanol (1:1) was spread uniformly over a sheet of 2.5 cm^2 nonwoven polyethylene cloth (Johnson and Johnson, USA), which was then applied to the back area of nude mice. The polyethylene cloth was fixed with Tegaderm adhesive dressing (3M, St. Paul, MN) and Fixomull stretch adhesive tape (Beiersdorf, Hamburg, Germany). At 2, 4, and 8 h after application, the mice were sacrificed and their skins were excised and analyzed for chrysin content using the same procedures as for the in vitro experiments.

HPLC Analytical Methods. The HPLC system for chrysin included an L-2130 pump, an L-2200 sample processor, and an L-2400 UV−visible detector from Hitachi (Tokyo, Japan). A 25 cm long, 4 mm inner

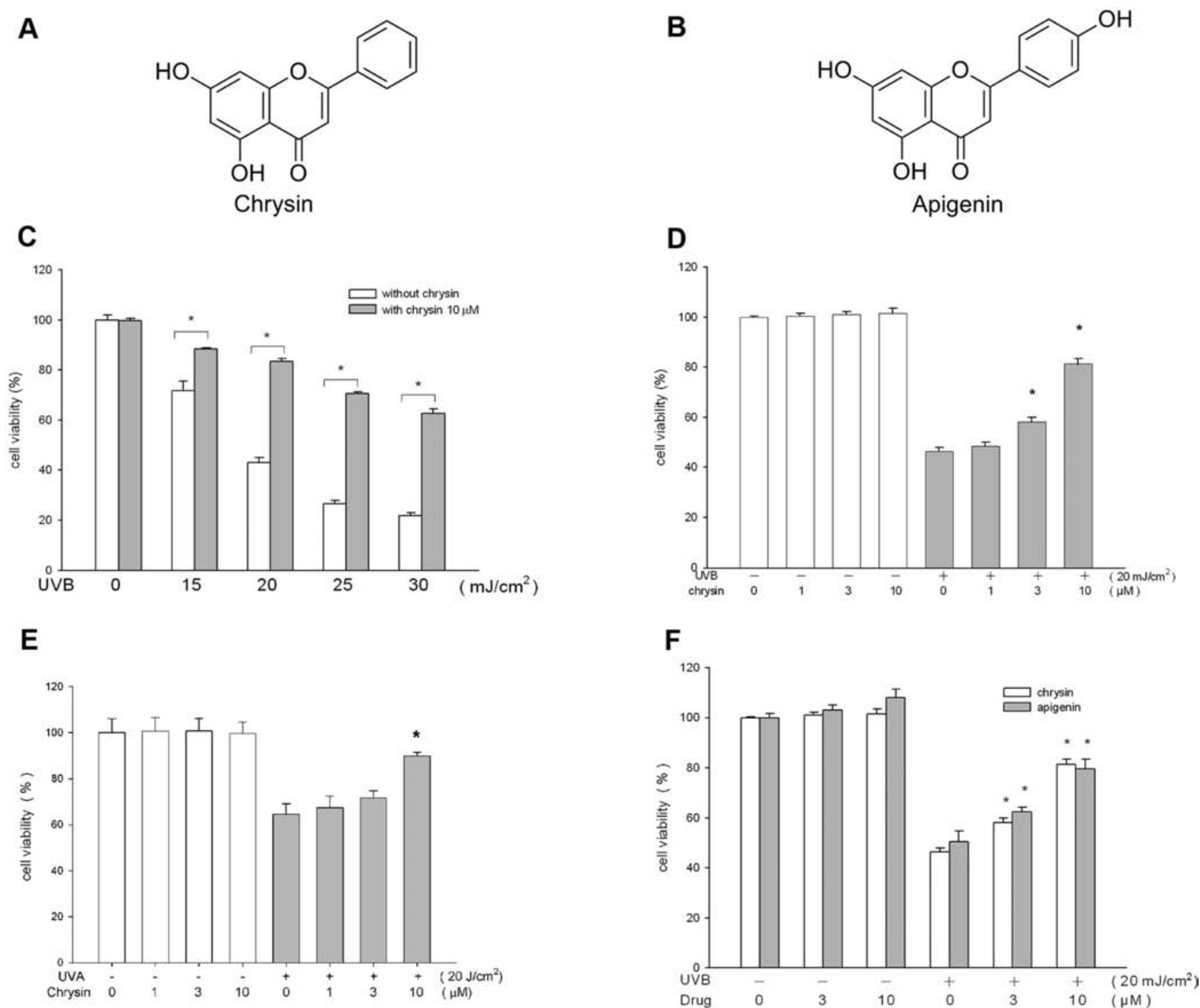

Figure 1. Chrysin attenuated UVA- and UVB-induced cell death in keratinocytes. Panels A and B illustrate the chemical structures of chrysin and apigenin. MTT assay was used to evaluate cell viability 24 h after treatments. (C) After pretreatment with chrysin, HaCaT keratinocytes were irradiated with UVB in increasing doses. (D) After pretreatment with chrysin in indicated concentrations, HaCaT keratinocytes were irradiated with UVB (20 mJ/cm^2). (E) Similarly, HaCaT keratinocytes were irradiated with UVA (20 J/cm^2) after pretreatment with chrysin at the indicated concentrations. (F) To compare the effects of chrysin and apigenin, HaCaT keratinocytes were irradiated with UVB after pretreatment with chrysin or apigenin at the same indicated concentrations. Results are expressed as the percentage of the control (mean ± SEM, $n = 3$). *, $p < 0.05$ versus irradiated cells without drug treatment.

diameter stainless steel RP-18 column (Merck, Darmstadt, Germany) was used as the stationary phase. The mobile phase was a mixture of methanol and pH 2.7 water adjusted by acetic acid (75:25) at a flow rate of 1 mL/min. The UV−visible detector was set at 264 nm.

In Vivo Bioengineering Evaluations. The method of drug application to nude mice was the same as described previously. After a 24 h application, the polyethylene cloth was removed, and the area of application was gently cleaned using a cotton wool swab. Thirty minutes after withdrawal of the drug, the extent of erythema, transepidermal water loss (TEWL), and pH were determined quantitatively using a colorimeter (Minolta Chroma Meter CM-503C, Yokohama Electrical Co., Japan), Tewameter (TM300, Courage & Khazaka, Germany), and pH meter (Skin-pH-meter, Courage & Khazaka). An adjacent untreated site was used as the baseline standard for each determination. The examinations were performed for three consecutive days.

Statistical Analysis. Data were expressed as the mean ± SEM. Means of groups of data were compared using the unpaired, two-tailed Student t test. A P value of <0.05 was considered to be statistically significant.

■ RESULTS

Chrysin Prevented Apoptosis Induced by UVA and UVB Irradiation in Keratinocytes. Panels A and B of Figure 1 show the chemical structures of chrysin and apigenin. Cell death of HaCaT keratinocytes, induced by UVB gradually increased with increasing irradiation dose. Pretreating cells with chrysin substantially enhanced the cell survival rate (Figure 1C). Pretreatment of cells by chrysin increased cell viability after UVB

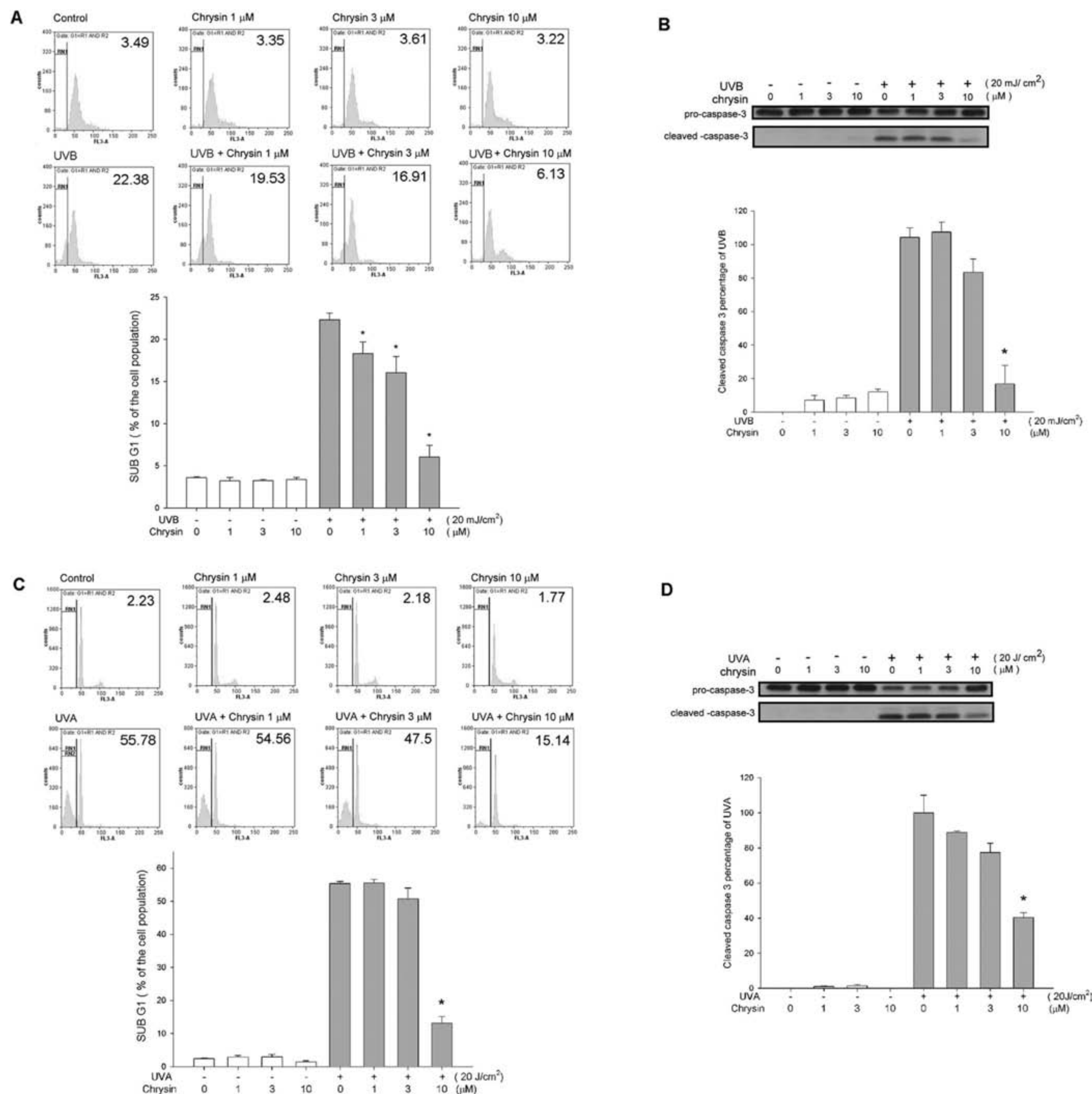

Figure 2. Blocking of sub G1 phase accumulation and activation of procaspase 3, caused by UVB and UVA irradiation, by chrysin. (A) HaCaT keratinocytes were irradiated with UVB after pretreatment with chrysin in increasing concentrations. Flow cytometry analyzed the sub G1 phase 6 h after UVB irradiation. (B) HaCaT keratinocytes were treated as in (A), and then Western blotting was used to evaluate procaspase 3 processing 8 h after UVB irradiation (upper panel). Quantification data are shown in the lower panel. (C) Similar to (A), the sub G1 phase was assayed after UVA irradiation. (D) Similar to (B), procaspase 3 cleavage was evaluated after UVA irradiation (mean ± SEM, $n = 3$). ∗, $p < 0.05$ versus irradiated cells without drug treatment.

irradiation (20 mJ/cm^2) in a dose-dependent manner (Figure 1D). Similarly, chrysin reversed cell death after UVA irradiation (20 J/cm^2) (Figure 1E). Comparison of the protective effects of chrysin with those of apigenin, another kind of flavonoid, demonstrated the comparable efficiency of chrysin in the prevention of UVB-induced cell death (Figure 1F). In the present study investigation into procaspase 3 processing and analysis of the cell cycle confirmed the modulation of UV-triggered apoptotic pathways by chrysin. As shown in Figure 2A,B, chrysin down-regulated the accumulation of cells in sub G1 phase and attenuated the cleavage of procaspase 3, caused by UVB irradiation, in a dose-dependent manner. Similarly, after pretreatment by chrysin, the sub G1 phase fraction and cleavage of procaspase 3 induced by UVA also decreased (Figure 2C,D).

Chrysin Decreased ROS Overproduction Triggered by UVA and UVB. Oxidative stress can mediate various harmful effects caused by UVA and UVB.[7] In this study, chrysin demonstrated ROS scavenging ability. As shown in Figure 3, 30 min

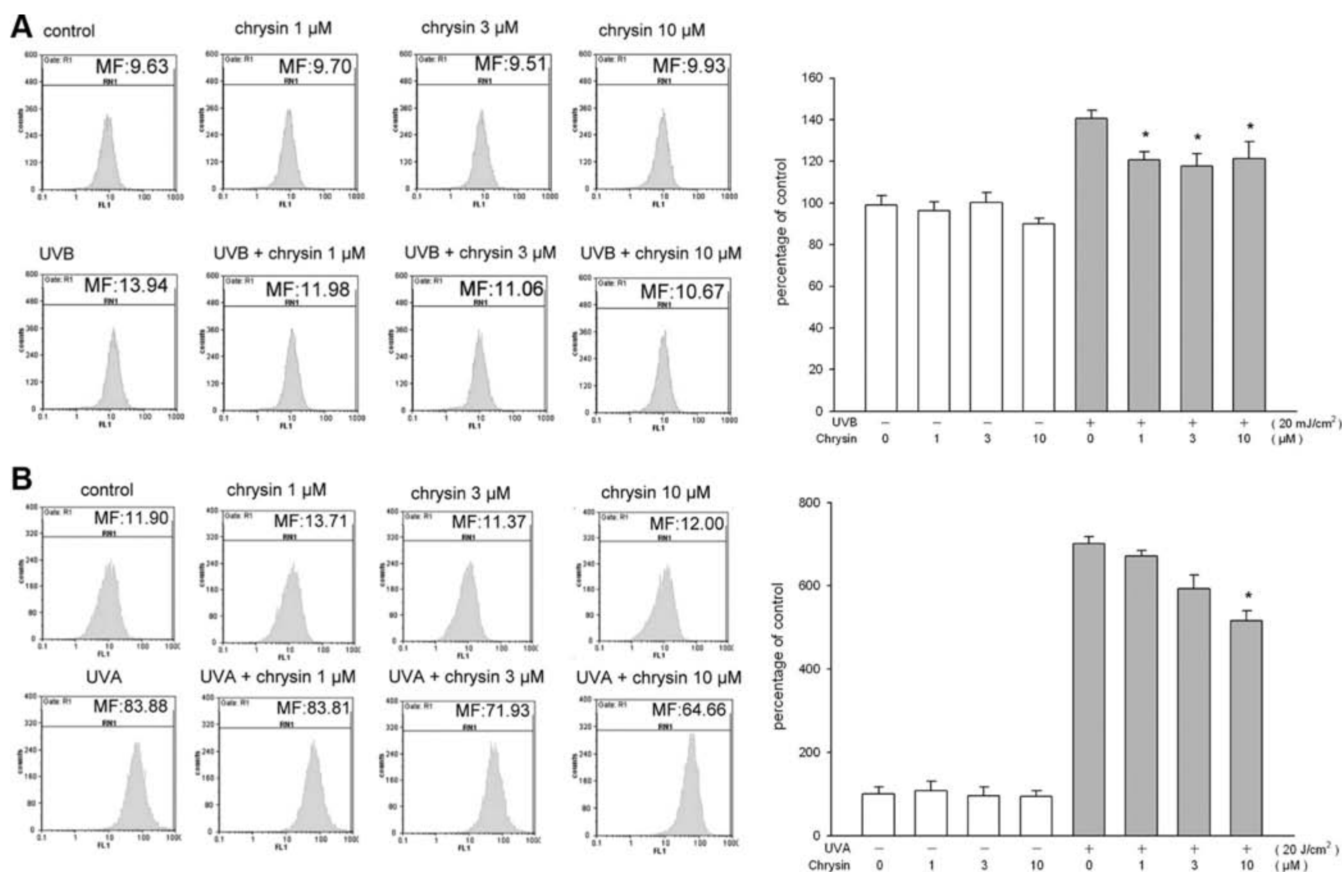

Figure 3. Inhibition of ROS production, triggered by UVA and UVB, by chrysin pretreatment in HaCaT keratinocytes. Flow cytometry was used to investigate ROS production in HaCaT keratinocytes 30 min after UV irradiation. (A) After pretreatment of chrysin at the indicated concentrations, cells were exposed to UVB. (B) Experimental procedures were similar to those for (A) but UVA was used for irradiation. Data are expressed as the percentage of the control (mean ± SEM, $n = 3$). *, $p < 0.05$ versus irradiated cells without drug treatment.

after UVA and UVB irradiation, flow cytometry detected increased ROS production in HaCaT keratinocytes. Compared with UVB, UVA displayed more prominent action on the enhancement of ROS production. Pretreatment with chrysin significantly suppressed ROS production induced by both UVA and UVB.

Chrysin Inhibited COX-2 Expression Caused by UVA and UVB. Previous research reported COX-2 induction by UVB and its critical roles in UV-induced pathogenesis.[10] The present study confirmed the dose-dependent induction of COX-2 by UVB irradiation after 48 h (Figure 4A). Pretreatment of HaCaT keratinocytes with chrysin before UV irradiation addressed the effects of chrysin on COX-2 induction. As shown in Figure 4B, chrysin inhibited the induction of COX-2 36 and 48 h after UVB irradiation. Increasing the chrysin concentration increasingly inhibited UVB-induced COX-2 expression (Figure 4C). Similarly, after UVA irradiation, chrysin inhibited the induction of COX-2 in a dose-dependent manner (Figure 4D).

Chrysin Regulated UVB- and UVA-Activated Signaling of MAP Kinases in HaCaT Keratinocytes. MAP kinase signaling is critically involved in UV-activated pathways and regulates various downstream effects in keratinocytes.[1] Subsequent to evaluation of the effects of chrysin on UV-irradiated keratinocytes described previously in this paper, this study investigated the possible signaling pathways modulated by chrysin. As shown in Figure 5A, ERK1/2, p38, and JNK phosphorylation were induced by UVB irradiation 30 min later. Chrysin pretreatment inhibited JNK phosphorylation in a dose-dependent manner, mildly decreased p38 phosphorylation, and barely affected ERK1/2 activation. Under the UVA irradiation, the signalings of ERK1/2, p38 and JNK were also obviously activated 30 min after irradiation. Pretreating HaCaT with chrysin inhibited JNK phosphorylation in a dose-dependent manner and mildly attenuated p38 and ERK1/2 phosphorylation (Figure 5B).

Chrysin Prevented the UVB-Induced Down-regulation of AQP-3. AQP-3 is crucial for the maintenance of cutaneous homeostasis,[12] and previous research has reported the regulation of AQP-3 expression by UV radiation.[11] Consequently, this study explored any possible beneficial effects of chrysin on the regulation of AQP-3 expression. As shown in Figure 6, UVB could down-regulate AQP-3 expression in HaCaT keratinocytes. Pretreatment with chrysin reversed this down-regulation in a dose-dependent manner. Quantification confirmed the significance of this effect.

Topical Application of Chrysin Demonstrated Efficient Percutaneous Absorption and No Skin Irritation. To evaluate chrysin's potential clinical application, the present study assessed the efficiency of percutaneous absorption and any cutaneous reaction after topical application of chrysin on mice. Using the Franz diffusion cell, the time-dependent cumulative absorption of chrysin from stripped mouse skin was efficient (Figure 7A). The permeate flux rate was also favorable for the stripped skin.

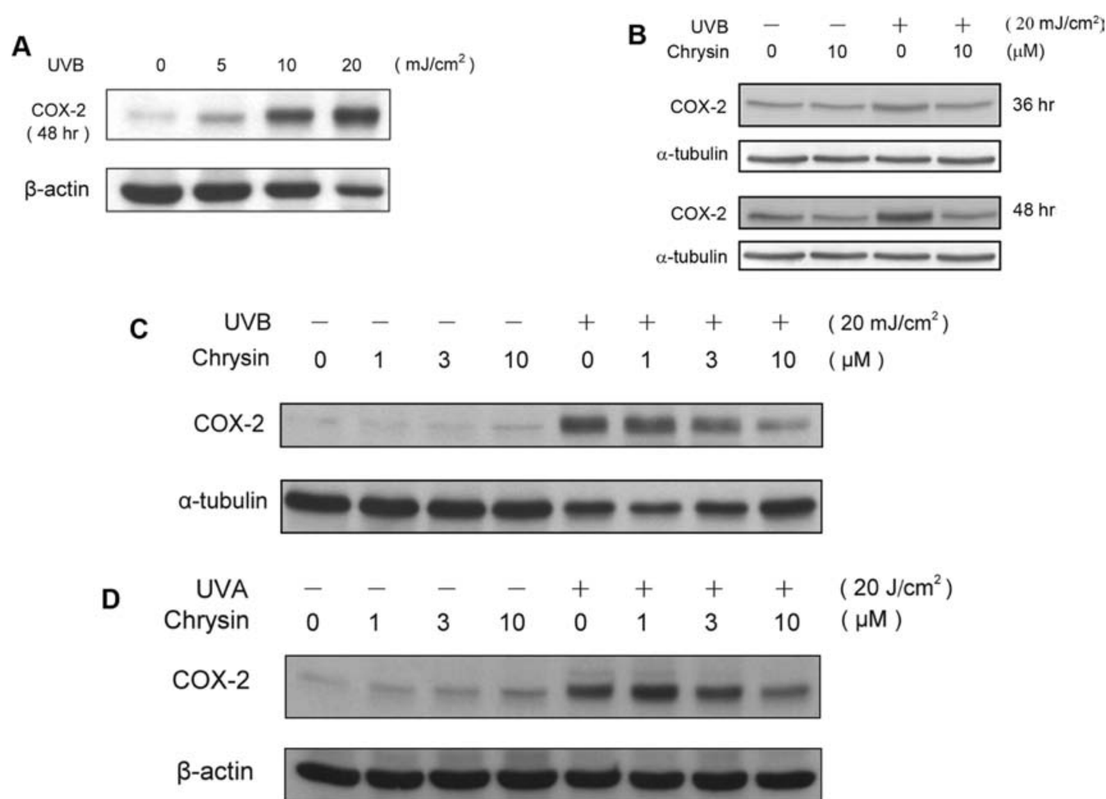

Figure 4. Chrysin decreased the expression of COX-2 induced by UVA and UVB in a dose-dependent manner. Western blotting was used to assay the changes in COX-2 expression after different treatments. (A) COX-2 expression in HaCaT keratinocytes was determined 48 h after irradiation with UVB in increasing doses. (B) HaCaT keratinocytes were pretreated with chrysin and then irradiated with UVB. Cell lysates were harvested 36 and 48 h after irradiation. (C) HaCaT keratinocytes were pretreated with different doses of chrysin and then irradiated with UVB. Cell lysates were collected 48 h after irradiation. (D) The experiment was similar to that in (C) but UVA was used for irradiation.

Analysis of the skin deposition of chrysin on stripped skin of nude mice showed acceptable results (Table 1). Twenty-four hours after the application of chrysin to nude mice, pH value, transepidermal water loss (TEWL), and erythema (a^*) did not demonstrate significant differences from untreated sites (Figure 7B). Analyzing the tissues from nude mice collected at indicated time points demonstrated that the chrysin skin deposition content in this in vivo model reached quite large amounts within 2 h (Table 2).

DISCUSSION

UV irradiation is a well-known environmental hazard causing various kinds of cutaneous damage. Use of botanical agents, such as flavonoids, to reduce solar UV-induced harmful effects has increasingly attracted interest.[26,27] However, compared with most agents photoprotective against UVB-induced damage in skin, a relatively small number of natural compounds reportedly protect against UVA-induced cytotoxicity.[22,28,29] In this study, chrysin protected keratinocytes against apoptosis caused by physiological doses of UVA and UVB radiation. This is potentially beneficial in maintaining the integrity of normal skin barrier function in response to UV exposure.

UV-induced apoptosis can be the consequence of DNA damage and activation of death receptors. Both pathways can subsequently go to apoptosis by activating the downstream effector protease, caspase 3.[6] The present study's results demonstrated that chrysin pretreatment attenuated UVA- and UVB-triggered processing of procaspase 3, reducing UV-induced apoptosis. In addition to photoprotective effects, previous research has described suppression of cleavage of procaspase 3 by chrysin and consequent inhibition of apoptosis in neuronal cells in response to apoptotic inducers such as tunicamycin.[30]

Multiple proapoptotic and antiapoptotic mechanisms regulate the responses of keratinocytes after UV irradiation. For example, JNK activation is essential for UV-induced apoptosis, but activation of ERK, NF-κB, PI3K/Akt, and the mammalian target of rapamycin (mTOR) complex can be the surviving pathways against UV-induced death signaling.[1,6,31] In the present study, chrysin barely affected ERK1/2 signaling, but prominently inhibited UVA- and UVB-activated JNK signaling, which might account for the observed attenuation of UV-induced apoptosis. However, further evaluation of the effects of chrysin on other surviving pathways, such as PI3K and NF-κB signaling, evoked by UV irradiation is needed.

UV-induced ROS production can cause various skin disorders by inducing DNA damage and mediating MAP kinase signaling.[1,6,7] Many flavonoids reportedly reduce ROS overproduction and inhibit the following adverse effects.[22,23,26] The present study disclosed that chrysin reduces UVA- and UVB-induced ROS production, which may contribute to the inhibition of JNK activation and downstream signaling, although the possibility of direct inhibition of JNK by chrysin cannot be excluded. However, although ROS is critically involved in apoptosis induced by UVA and UVB, many factors besides ROS may also account for UV's proapoptotic effects. As for UVB-induced apoptosis, UVB can induce clustering and activation of death receptor (CD95), which

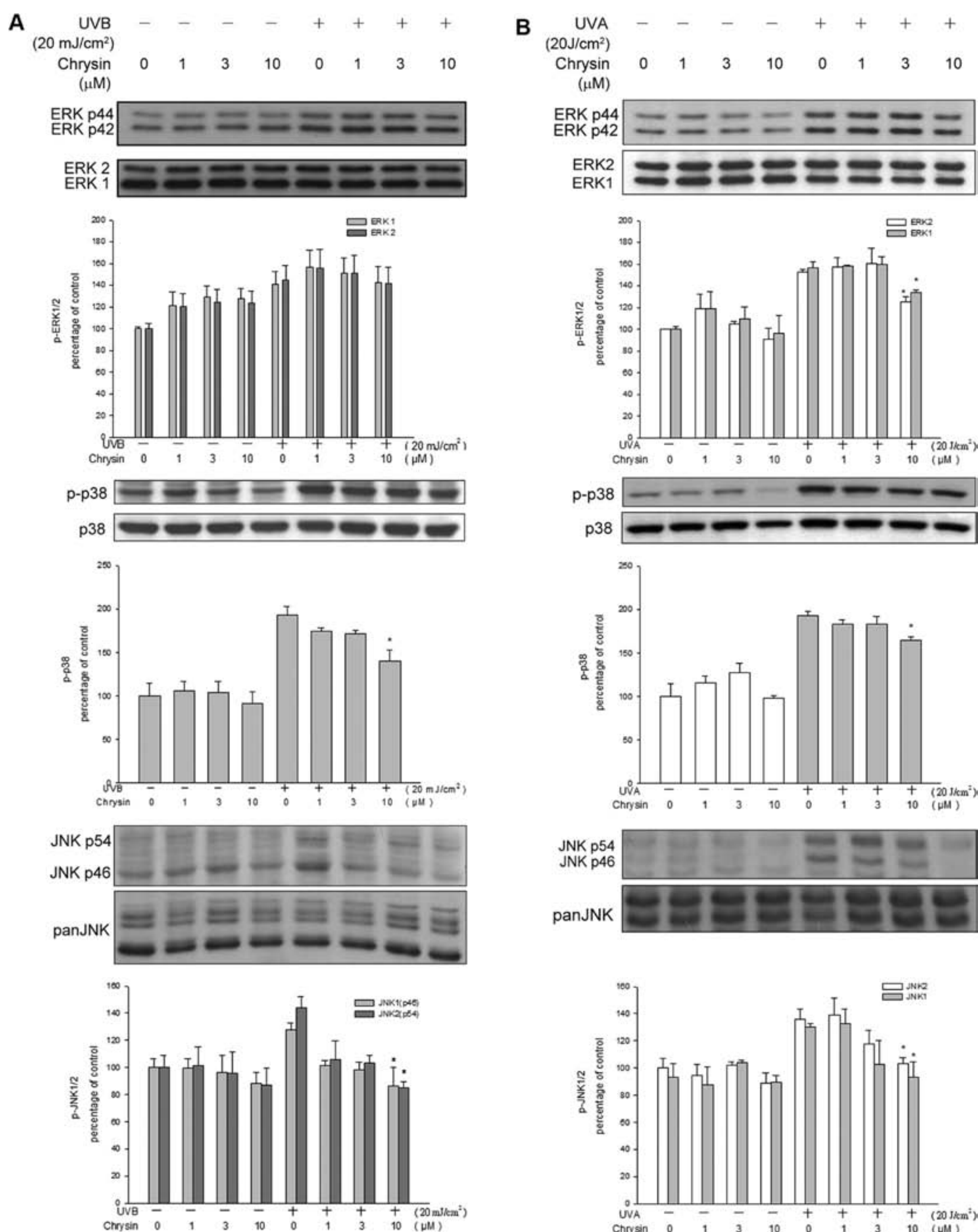

Figure 5. Chrysin modulated the UVA- and UVB-evoked signaling of MAPKs in HaCaT keratinocytes. Western blotting was used to evaluate the changes in phosphorylated ERK1/2, p38, and JNK expression after UV irradiation. (A) HaCaT keratinocytes were pretreated with different doses of chrysin and irradiated with UVB. Thirty minutes after irradiation, cell lysates were harvested for analysis. Quantification of changes is shown as graphs. (B) The experiment was similar to that in (A) but UVA was used for irradiation. Quantification evaluated the changes in signals. Data are expressed as the percentage of control (mean ± SEM, $n = 3$). ∗, $p < 0.05$ versus irradiated cells without drug treatment.

can, in turn, lead to apoptosis independently of ROS production.[32] To date, studies of the molecular mechanisms of UVA-induced apoptosis in keratinocytes are fewer than those of UVB, but previous research has described the trigger of rapid apoptosis by UVA via activation of CD95 death pathways in HL-60 cells.[33] Induction of ROS by UVA could also be related to survival signaling, and attenuation of ROS production by 3-*O*-caffeoyl-1-methylquinic acid (MCGA3) enhanced the UVA-induced apoptosis in HaCaT cells.[34] The previously mentioned findings suggest that ROS production may not entirely be responsible for UVA- and UVB-induced apoptosis. Chrysin may, therefore, regulate other antiapoptotic mechanisms in addition to ROS scavenger activity. In addition, UVA can penetrate the skin deeply and powerfully, causing oxidative damage within the range of the solar spectrum.[5,22,28] Future studies may, therefore, explore the effects of chrysin on UVA-induced responses in dermal cells, such as dermal fibroblasts, to evaluate the protective function of chrysin in UV-induced dermal injuries such as photoaging.

COX-2 plays a central role in diverse skin disorders including cutaneous inflammation, nociception, wound healing, and tumorigenesis. Inhibition of COX-2 expression is a novel pharmacological approach to treat skin diseases.[4,8] Different botanical extracts inhibit UV-induced COX-2 expression, such as curcumin, apigenin, and anthocyanins.[2,10,31] Khan et al. demonstrated that chrysin inhibits diethylnitrosamine-induced COX-2 expression during hepatocarcinogenesis.[21] However, chrysin also reportedly suppresses lipopolysaccharide (LPS)-induced prostaglandin E2 formation but does not inhibit LPS-induced COX-2 expression in RAW 264.7 cells.[18] The present study showed that chrysin dose-dependently suppressed UVA- and UVB-induced COX-2 expression in HaCaT keratinocytes. Although several signaling molecules such as p38, ERK, and PI3kinase/Akt (but not JNK) reportedly regulate COX-2 expression in HaCaT cells,[31] findings from a previous study by Cho et al. indicated that inhibition of JNK signaling could decrease UVB-induced COX-2 expression.[2] Similarly, prior investigations demonstrated that p38 and JNK signaling regulate UVA-induced COX-2 expression in HaCaT cells.[35−37] In the present study's results, chrysin markedly inhibited UVA and UVB-activated JNK phosphorylation but mildly affected p38 phosphorylation, suggesting chrysin may inhibit UV-induced COX-2 expression via either or both pathways of p38 and JNK activation. Previous research has also reported oxidative stress-dependent up-regulation of COX-2, indicating that reduction of ROS and related downstream signaling by chrysin may mediate inhibition of COX-2 expression.[38]

Prior investigations described the importance of AQP-3 in skin homeostasis and barrier function.[12,13] The development of drugs that target AQP-3 expression, therefore, shows future promise. UVB can down-regulate the expression of AQP-3 in skin;[11] therefore, reversing the diminution of AQP-3 expression by UVB is important in the prevention of solar damage. As previously described, retinoic acid enhances AQP-3 expression in normal skin both in vitro and ex vivo[39] and attenuates the UV irradiation-induced down-regulation of AQP-3.[11] Owing to the

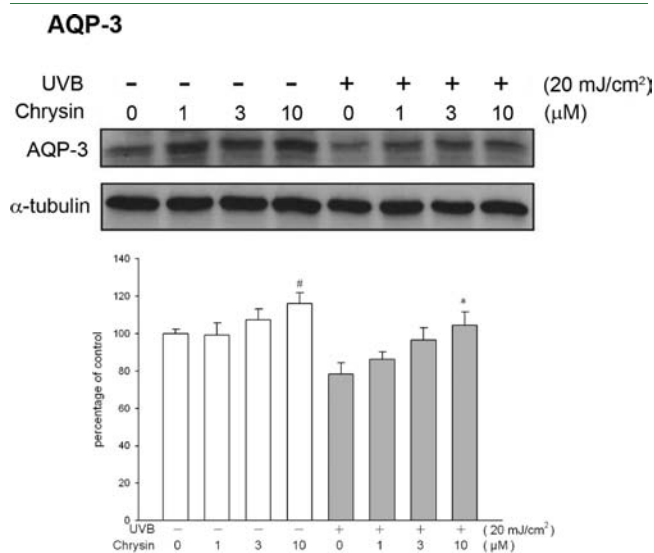

Figure 6. Chrysin reversed UVB-induced reduction in AQP-3 expression. HaCaT keratinocytes were pretreated with different doses of chrysin and irradiated with UVB. Cell lysates were collected after 24 h and analyzed using Western blotting. Quantification also evaluated the changes in AQP-3. Data are expressed as the percentage of control (mean ± SEM, $n = 3$). #, $p < 0.05$ versus the control; ∗, $p < 0.05$ versus irradiated cells without drug treatment.

Table 1. In Vitro Permeation Data of Chrysin from PEG400/Ethanol (1:1) Vehicle[a]

skin type	flux (nmol/cm²/h)	skin deposition (nmol/mg)
intact skin	4.56 ± 2.43	0.25 ± 0.10
stripped skin	16.27 ± 5.37	0.84 ± 0.37
cellulose membrane	24.04 ± 6.27	—[b]

[a] Each value represents the mean ± SD ($n = 4$). As the experiment in Figure 7A, the flux of permeation rate was determined by the curve of cumulative amount of released chrysin at different time points. The cellulose membrane or intact or tape-stripped skin of nude mice was collected, lysed, and subjected to analysis of the content of chrysin. [b] —, not determined.

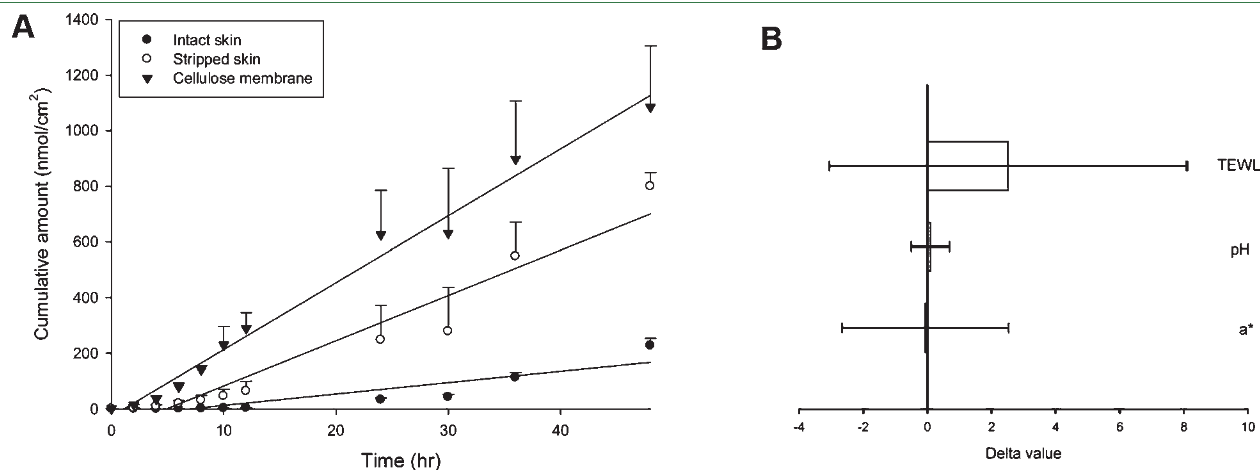

Figure 7. Transepidermal absorption and safety of chrysin were evaluated in animal studies. (A) The cellulose membrane, intact skin of nude mice, and tape-stripped skin of nude mice were used in the device of percutaneous absorption. The in vitro cumulative amount of chrysin was detected at indicated time points. (B) At 24 h after the application of chrysin in PEG400/ethanol (1:1) on nude mice, the transepidermal water loss (TEWL), pH, and erythema (a^*) values were determined. The Delta value indicated the value of the treated site minus the value of an adjacent untreated site. Data are presented as the mean ± SEM, $n = 3$.

Table 2. In Vivo Skin Deposition of Chrysin from PEG400/Ethanol (1:1) Vehicle after Application on the Back Skin of Nude Mice[a]

application duration (h)	skin deposition (nmol/mg)
2	0.48 ± 0.15
4	0.64 ± 0.27
8	0.41 ± 0.26

[a] Each value represents the mean ± SD ($n = 5$). Chrysin in PEG/ethanol was applied on the back of nude mice. At indicated time points, mice were sacrificed, and the tissues from treated areas of mice were subjected to detection of chrysin content.

involvement of ERK activation and ROS in suppressed AQP-3 expression caused by UVB irradiation,[11] chrysin's ROS scavenger ability may account for its effect on the recovery of AQP-3 expression after UVB irradiation. The ability of chrysin to reverse the diminution of AQP-3 expression by UV implies that chrysin is potentially useful in the restoration of impaired barrier function by UV and could provide a valuable photochemopreventive agent for use in daily life.

Further to determining the distinct functions of chrysin, the present study evaluated the possibility of its clinical application. The transepidermal absorption studies illustrated the efficient penetration of chrysin into skin when topically used. Evaluation of irritation after topical application to mouse skin showed chrysin's safety and suggested its potential for application in a clinical setting. Further studies may include the investigation of effects of chrysin on animals under UV irradiation, in order to establish its protective function on UV-induced damage in vivo.

The present study demonstrated that chrysin can ameliorate various kinds of skin damage caused by UVA and UVB, including apoptosis, ROS overproduction, COX-2 induction, and down-regulation of AQP-3. Animal studies confirmed the efficiency and safety of chrysin's topical application. The benefits identified demonstrate the potential use of chrysin, a natural compound in common foods, in the prevention of UV-induced deleterious effects. Its use as a photochemopreventive product in daily life shows future promise.

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ABBREVIATIONS USED

UV, ultraviolet; COX-2, cyclooxygenase 2; ROS, reactive oxygen species; AQP-3, aquaporin 3; ERK, p42/44 extracellular signal regulated kinase; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase.

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