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Baicalin Scavenged Reactive Oxygen Species and Protected Human Keratinocytes Against UVB-induced Cytotoxicity

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Abstract. *Ultraviolet B (UVB), with a wavelength of 280-320 nm, represents one of the most important environmental factors for skin disorders, including sunburn, hyperpigmentation, solar keratosis, solar elastosis and skin cancer. Therefore, protection against excessive UVA-induced damage is useful for prevention of sunburn and other human diseases. Baicalin, a major component of traditional Chinese medicine Scutellaria baicalensis, has been reported to possess antioxidant and cytostatic capacities. In this study, we examined whether baicalin is also capable of protecting human keratinocytes from UVB irradiation. The results showed that baicalin effectively scavenged reactive oxygen species (ROS) elevated within 4 h after UVB radiation and reversed the UVB-suppressed cell viability and UVB-induced apoptosis after 24 h. Our results demonstrated the utility of baicalin to complement the contributions of traditional Chinese medicine in UVB-induced damage to skin and suggested their potential application as pharmaceutical agents in long-term sun-shining injury prevention.*

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Ultraviolet (UV) light, which can be further divided into UVA (320-400 nm), UVB (280-320 nm) and UVC (200-280 nm), is one of the harmful environmental factors causing skin disorders, including sunburn, hyperpigmentation, solar keratosis, solar elastosis and skin cancer (1). UVA and UVB are the main components of sunlight reaching the earth surface, with UVB radiation being a potent mutagen and responsible for the induction of skin melanoma and non-melanoma, as well as sunburn and photo-aging. UVC radiation is also a mutagen but it is absorbed by the stratospheric ozone layer and, thus, the damage effect of UVA to skin is tremendously weaker than UVB radiation (2, 3). UVB damages our genome through photo-chemical reactions and literature, in the past two decades, has demonstrated the importance of UVB-induced reactive oxygen species (ROS) with the reaction of skin cells (4, 5). However, it is still not clear, to date, how ROS are generated after UVB irradiation and how could this action be prevented by natural compounds.

The famous traditional Chinese medicine *Scutellaria* (Huang-Qin) is especially highly rich in flavonoid contents, which have been reported to have antitumor capacity (6, 7). In the literature, these flavones, isolated from the roots of *Scutellaria*, have antioxidant (8), anti-viral (9-11), anti-thrombotic (12, 13) and anti-inflammatory (14) properties. In 2013, we reported that baicalin (7-glucuronic acid, 5,6-dihydroxy-flavone), one of the major flavones in *Scutellaria* (15), could suppress cytotoxicity and ROS induced by UVC (16). The present study aims at understanding the UVB-induced cytotoxicity, apoptosis and ROS generation. It also questions whether baicalin is able to suppress UVB-induced cytotoxicity and ROS production.

Materials and Methods

Chemicals. All chemicals and solvents used throughout this study, such as baicalin, dimethyl sulfoxide (DMSO), propidium iodide (PI), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were obtained from Sigma Chemical Co. (St. Louis, MO, USA) and Aldrich Chemical Co. (Milwaukee, WI, USA). Dulbecco's modified Eagle's medium and penicillin/streptomycin were obtained from GIBCO/BRL Life Technologies (Cambrex, Walkersville, MD, USA). T4 UV endonuclease V was purchased from Epicentre Technologies (Madison, WI, USA). Formamido-pyrimidine-DNA glycosylases (Fpg) and endonuclease III were purchased from Trevigen (Gaithersburg, MD, USA).

Cell culture. Keratinocytes in human skin epidermis are the main target cells for UVB radiation. Typical human keratenocyte cells, HaCaT, were obtained from American Type Culture Collection (Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 mM non-essential amino acid, 2 mM glutamate, 100 U/ml penicillin and 100 μg/ml stryptomycin. The cultures were incubated at constant 37˚ and humidified atmosphere with 5% CO_2. HaCaT cells were sub-cultured every 2-3 days before they were fully confluent to obtain an exponential growth.

Cell viability examination. For determining cell viability, the MTT assay was performed as previously published (16-18). Briefly, HaCaT cells were plated into 24-well plates at the density of 3×10^4 cells/well, grown for another 24 h and, then, treated with 0.1% DMSO or different concentrations of baicalin. After the baicalin treatment, MTT was added to each well at a final concentration of 0.5 mg/ml and the mixture of MTT and cells was further incubated in 37˚ for 4 h. The viable cell number was directly proportional to the production of formazan following solubilization with isopropanol. Subsequently, the color intensity was measured at 570 nm in a Multiskan MS ELISA reader (Labsystems, Helsinki, Finland). The experiments were performed at least in triplicate.

Cell cycle distribution determination. Approximately 2×10^6/ml HaCaT cells were seeded in 10-cm dishes and treated with 0-200 μM baicalin for a 24-h period. In the UVB-irradiation-related experiments, cells were pre-treated with baicalin for 2 h, irradiated and, then, re-incubated with baicalin for 24 h. Finally, the cells were harvested and fixed gently with 70% ethanol, washed twice with PBS and, subsequently, incubated with PI buffer (4 μg/ml PI, 0.5 μg/ml RNase and 1% Triton X-100 in PBS) for 30 min in the dark at room temperature. The cells were filtered through a 40-μm nylon filter and the PI stained cells were analyzed for cell cycle distribution and appearance of sub-G1 phase by using a FACS Calibur instrument (BD Biosciences, San Jose, CA, USA) equipped with Cell Quest software as described previously (16-18).

UVB density measurement and UVB exposure. After using the UV light crosslinker Spectrolinker XL-1000 (Spectronics Co., Westburg, NY, USA), the UV dose was measured by a sensor in the UVB light box. All cells were washed with phosphate buffered saline (PBS), drained in a dish, exposed to UVB radiation as indicated and immediately returned to incubation medium.

ROS measurement. About 2×10^5 HaCaT cells/well in 12-well plates were pre-treated for 2 h with 0-200 μM baicalin, then, irradiated by UVB and re-treated with the same dose of baicalin for 0 to 4 h to determine the resulting ROS production. After cells were incubated for various time periods, all the cells in each treatment were harvested, washed twice by PBS and re-suspended in 500 μl of dichloro-dihydro-fluorescein diacetate ((DCFHDA); Sigma Chemical Co.) (final concentration of 10 μM) in dark for 30 min. Then all samples were analyzed immediately by flow cytometry as previously described (16).

Statistical methodology. In this study, analysis of variance (ANOVA) was employed and data were expressed as mean±SEM. Student's *t*-test was used in two-group comparisons. $p<0.05$ was considered to be statistically significant.

Results

Baicalin can effectively reverse the cytotoxicity of UVB on HaCaT cells. First, the cytotoxic effects of UVB alone on HaCaT cells were investigated. MTT results showed that 5, 10, 20, 40, 60 and 80 mJ/cm^2 UVB irradiation induced significant loss of cell viability of 4.5, 15.3, 25.5, 55.0, 77.3 and 84%, respectively (Figure 1A). Subsequently, in order to investigate the cytotoxic effects of baicalin alone on HaCaT cells, baicalin, at a concentration of 0 to 200 μg/ml, was added for 26 h and cell viability was analyzed by the MTT assay. The data showed that treatment of baicalin alone for 26 h may induce 7.2 and 8.2% of decreased cell viability at 150 and 200 μg/ml; no obvious cytotoxicity of baicalin was observed under the dose of 100 μg/ml (Figure 1B). Then, HaCaT cells were pre-treated with 0 to 200 μg/ml of baicalin for 2 h, irradiated with UVB at 40 and 60 mJ/cm^2 and, then, immediately re-incubated with the same dose of baicalin for another 24 h. MTT analysis showed that 40 mJ/cm^2 UVB irradiation induced 56.0% of reduced cell viability; however, 75 μg/ml of baicalin started to reversing the UVB-induced cytotoxicity, whereas 200 μg/ml of baicalin could reinstate cell viability almost to the level of non-UVB treated control cells (Figure 1B). When using 60 mJ/cm^2 UVB irradiation, an induction of 79.3% of reduced cell viability was observed. In this case, 50 μg/ml of baicalin started reversing UVB-induced cytotoxicity, while 200 μg/ml of baicalin reinstated cell viability to the extent of 61.8% of intact HaCaT cells (Figure 1B).

Baicalin can effectively reverse the UVB-induced apoptosis of HaCaT cells. HaCaT cells were pre-treated with baicalin, exposed to 0 to 80 mJ/cm^2 of UVB, re-treated with baicalin for another 24 h and harvested to determine the alteration in cell cycle distribution by evaluating the appearance of typical sub-G_1 (apoptosis) percentages using flow cytometry. The typical sub-G_1 phase in each treatment was calculated as % of the overall number of cells, with the final results being presented in Figure 2A. Our findings indicated that 10, 20, 40,

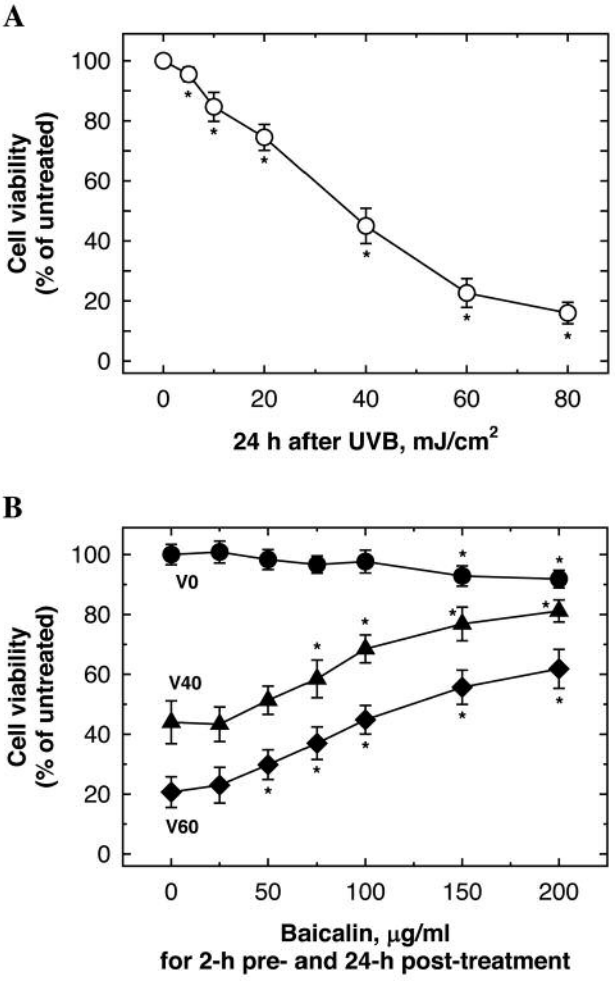

Figure 1. *Baicalin reversed UVB-induced cytotoxicity of HaCaT cells. (A) HaCaT cells were irradiated with 0-80 mJ/cm^2 UVB, harvested and analyzed for cell viability by MTT assay. *p<0.05, with versus without UVB irradiation; (B) HaCaT cells were pre-treated for 2 h with 0-200 μg/ml baicalin, irradiated with UVB 0 (●), 40 (▲) or 60 (◆) mJ/cm^2 and post-treated with the same dose of baicalin for another 24 h. Subsequently, cell viability was analyzed by the MTT assay. *p<0.05, with versus without baicalin treatment.*

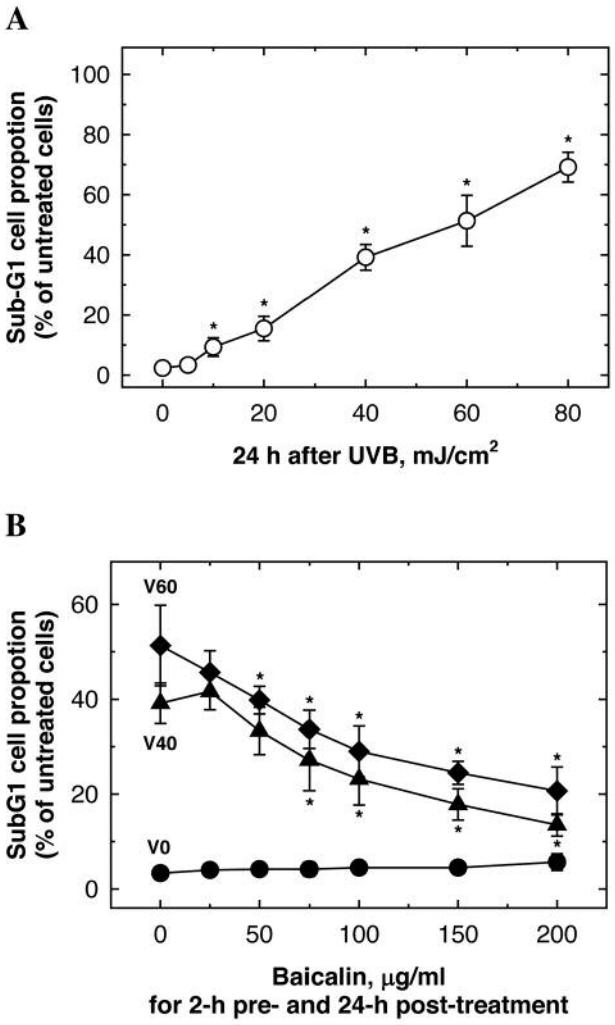

Figure 2. *Baicalin effectively reversed the UVB-induced apoptosis of HaCaT cells. (A) HaCaT cells were treated with 0-80 mJ/cm^2 UVB and the harvested cells were examined and analyzed for cell cycle distribution and sub-G_1 (apoptosis) by flow cytometry as described in Materials and Methods. Each point represents the mean±SD of three experiments. (B) HaCaT cells were pre-treated with 0, 25, 50, 100, 150 μg/ml baicalin for 2 h, irradiated with UVB 0 (●), 40 (▲) or 60 (◆) mJ/cm^2 and post-treated with another 24 h. Subsequently, cell cycle distribution and sub-G1 (apoptosis) were examined by flow cytometry. Each point represents the mean±SD of three experiments.*

60 and 80 mJ/cm^2 of UVB induced apoptosis to the level of 9.3, 15.5, 39.2, 51.3 and 69.2% of all cells after 24 h, respectively (Figure 2A). Control experiments showed that baicalin alone at 25 to 200 μg/ml could not, as expected, induce HaCaT cell apoptosis (Figure 2B). The quantitative data showed that the apoptotic effect induced on HaCaT cells by UVB 40 and 60 mJ/cm^2 could be reversed dose-dependently by baicalin when in the range of 75 to 200 μg/ml, while the apoptotic effect induced by UVB 40 and 60 mJ/cm^2 could be reversed dose-dependently by baicalin from 50 to 200 μg/ml (Figure 2B).

Baicalin can effectively suppress the UVB-induced ROS in HaCaT cells. Exposure to 40 and 60 mJ/cm^2 of UVB could induce a rapid increase of ROS in HaCaT cells within 0.5 h and reach a peak at 2 h with a sustained high ROS level up to 8 h (Figure 3A). At 24 h, ROS production faded to non-significant levels compared with basal levels of intact HaCaT cells (data not shown). Based on these findings, we pre-treated the HaCaT cells with baicalin for 2 h before UVB irradiation and post-treated them at the same dose for 4 h to

investigate the effects of baicalin on UVB-induced ROS in HaCaT cells. The results showed that baicalin could effectively suppress the 40 and 60 mJ/cm^2 UVB-induced ROS in HaCaT cells in a dose-dependent manner (Figure 3B).

Discussion

Traditional Chinese medicine has been shown to have a well-established effect in clinical studies, displaying a broad range of clinical effects, including alleviation of cancer-associated symptoms, prolonging survival rates, decreasing treatment-related toxicity and preventing proliferation, recurrence and/or metastasis of cancer cells (19-23). Mounting evidence from *in vivo* and *in vitro* experiments has revealed that baicalin has positive effects on improving microcirculation, eliminating high ROS status, anti-inflammation and anti-carcinogenesis (13, 24-26). In this study, we examined whether (i) baicalin could protect HaCaT cells against UVB irradiation and (ii) the underlying molecular mechanisms was *via* the suppression of ROS induction. In the literature, baicalin has been documented as having an inhibitory effect on UVB-induced photo-damage *via* blocking the relevant cytokine secretion and reducing the expression of several genes, including *p53, p21, c-fs, PCNA* and *RPA* (5, 27, 28). Earlier studies, conducted in our laboratory, have shown that baicalin can reduce the increased apoptosis rate, ROS production and formation of cyclobutane pyrimidine dimers (CPDs) and oxidative DNA adducts (16). In general, there are three main end-points of UVB-induced cellular events that were examined herein with the use of baicalin: a) cell viability; b) programmed cell death, apoptosis; and c) production of ROS. As for the first part, the UVB-suppressed cell viability was reversed by baicalin dose-dependently (Figure 1). As for the second part, UVB-induced apoptosis was also reversed by baicalin dose-dependently (Figure 2). As for the third part, UVB-induced rapid production of ROS was also reduced by baicalin in a dose-dependent manner (Figure 3).

Concerning the effects of baicalin on UVB-suppressed cell viability, we found that 40 and 60 mJ/cm^2 UVB caused 56.0 and 79.3% loss of cell viability of HaCaT cells (Figure 1A), which was found to be reversed by 2-h pre-treatment and 24-h post-UVB irradiation treatment of baicalin at doses of 75 and 50 μg/ml, respectively (Figure 1B). At similar dose levels, baicalin was capable of rescuing UVB-damaged HaCaT cells from undergoing apoptosis (Figure 2B). With regard to UVB-induced ROS, UVB irradiation could induce intracellular ROS production within 0.5 h, reaching peak levels at 2h (Figure 3A) that were, however, reduced by balicalin dose-dependently (Figure 3B). The results strengthen the possibility that UVB triggers induction of ROS in HaCaT cells, which may cause oxidative damage to cell components, including genome DNA, RNA, proteins, lipids and small molecules. The reduction of cytotoxicity and

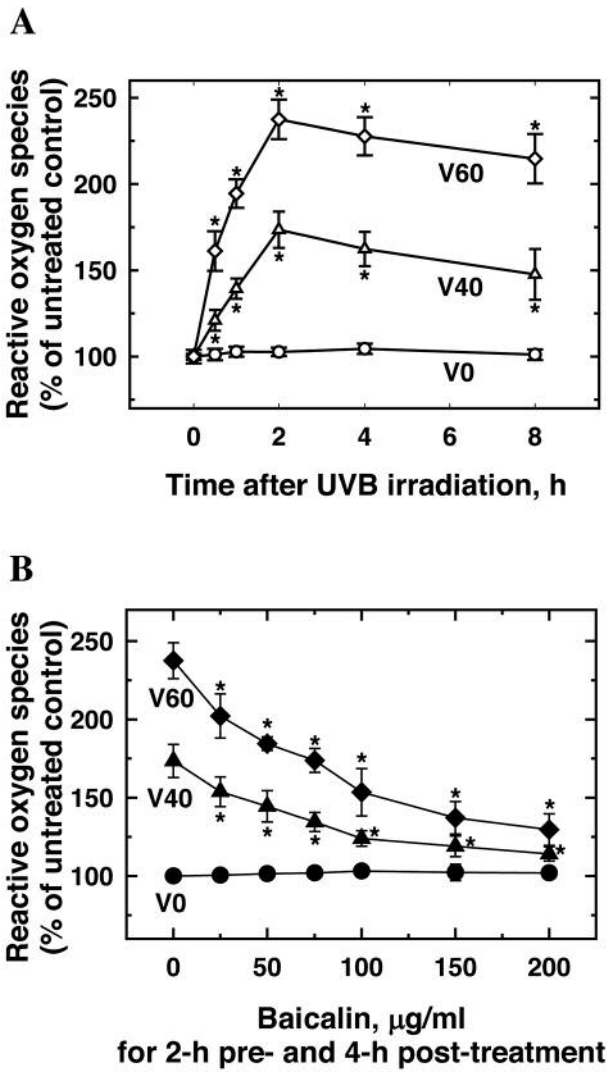

Figure 3. *Baicalin effectively reduced the UVB-induced reactive oxygen species (ROS) in HaCaT cells. (A) HaCaT cells were irradiated with 0 (○), 40 (△) and 60 (◇) mJ/cm^2 UVB, harvested and analyzed for ROS production. *p<0.05, with versus without re-incubation after UVB irradiation; (B) HaCaT cells were pre-treated for 2 h with 0-200 μg/ml baicalin, irradiated with UVB 0 (●), 40 (▲) or 60 (◆) mJ/cm^2 and post-treated with the same dose of baicalin for 4 h. Harvested cells were analyzed for ROS production. *p<0.05, with versus without baicalin treatment.*

apoptosis levels might be partly due to the property of the strong antioxidant activity of baicalin toward ROS. The mechanism of UVB-induced ROS production has been reported to be triggered by up-regulation of calcium in HaCaT cells (4). It remains, however, to clarify the detailed mechanism(s) by which baicalin suppresses the influx of calcium from extracellular space or the release of calcium from intracellular storage. Also, further studies are required

to investigate whether baicalin has any effect on the DNA damage/repair processes, such as production and removal of CPDs and/or oxidative adducts. In addition, it is uncertain, with respect to the source of UVB-induced ROS, whether the effects observed come from activation of NADPH oxidase (Nox), xanthine oxidase (XOD) or respiratory chain-chain reactions in mitochondria. Treatment with baicalin alone, up to 200 μg/ml, was non-toxic to HaCaT cells (Figures 1 and 2) and did not induce ROS (Figure 3). The non-toxicity of baicalin to HaCaT cells is consistent with that of Min's team and our previous findings (5, 16). Thus, baicalin, under the dose of 200 μg/ml, appears harmless to skin cells.

In conclusion, this study indicated that baicalin has UVB-protective capacity *via* reducing UVB-induced HaCaT ROS production, cell cytotoxicity and apoptosis.

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