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Anti-inflammatory effects of prunin on UVB-irradiated human keratinocytes

Eun Ju Na[1] and Ji Young Ryu[2*]

Abstract

Background: The flavonoid prunin is a flavanone glycoside found in cherry trees, including the flowering cherry *Prunus yedoensis Matsumura* (Rosacea). Although this compound has been studied for its antioxidant, anti-bacterial, and blood-sugar-lowering effects, no studies address its use in cosmetics. This study investigates whether prunin exhibits anti-inflammatory effects in cells exposed to ultraviolet B (UVB) radiation.

Methods: The effects of prunin were assessed by measuring cell viability using the water-soluble tetrazolium salt-1 assay and free radical damage using the dichlorofluorescein diacetate assay as well as by quantitative real-time PCR.

Results: UVB-induced decrease in cell viability diminished by pretreatment with prunin in a concentration-dependent manner. Intracellular reactive oxygen species (ROS) quantitative analysis revealed that the expression of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which is associated with the inflammatory response, and mRNA expression of interleukin-6 (*IL-6*), interleukin-8 (*IL-8*), cyclooxygenase-2 (*COX2*), tumor necrosis factor-alpha (*TNF-α*), and protease-activated receptor (*PAR2*) decreased with prunin pretreatment in a concentration-dependent manner.

Conclusions: Prunin increased the survival rate of UVB-treated human keratinocyte HaCaT cells. Prunin protected the HaCaT cells, eliminated ROS, and demonstrated anti-inflammatory effects. Thus, prunin is worthy of investigation for use as a cosmetic ingredient that protects the skin and has anti-inflammatory effects.

Keywords: Prunin, HaCaT keratinocyte, Anti-inflammation, Reactive oxygen species, Ultraviolet B

Background

The skin is put under oxidative stress when UV radiation induces the formation of reactive oxygen species (ROS), which damage DNA, lipids, and proteins (Devasagayam and Kamat 1994). Ultraviolet light acts as an external stress-stimulating factor, which increases the expression and activity of 11β-hydroxysteroid ehydrogenase type 1 (11β-HSD1), an enzyme that converts ROS and inactive cortisone to active cortisol in keratinocytes (Itoi et al. 2013).

Increased oxidative stress in the skin leads to inflammatory responses such as erythema, edema, and fever. These responses attempt to restore and regenerate wounds caused by chemical, physical, and bacterial assaults (Greaves and Sondergaard 1970; Hruza and Pentland 1993). During an inflammatory response, immune cells such as monocytes and macrophages are stimulated to express inflammation-related genes through the activation of transcription factors. Macrophages express the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in response to stimuli such as lipopolysaccharide and interferon-γ. NF-κB induces inflammatory responses by producing nitric oxide synthase, nitric oxide, prostaglandins, and cyclooxygenase-2 (COX2) (Nathan 1987). NF-κB is usually bound to inhibitor kappa B (IκB), which maintains the inactive state. When exposed to external stimuli such as UV light, IκB is rapidly phosphorylated and degraded (Maziere et al. 1999; Nomoto et al. 2001; Gomez-Nicola et al. 2010; Baeuerle and Henkel 1994). After NF-κB separates from IκB and becomes activated, it migrates to the nucleus, where it increases the expression of genes that induce inflammatory responses, including interleukin-6 (*IL-6*), interleukin-8 (*IL-8*), tumor necrosis factor-alpha (*TNF-α*), and *COX2* (Baeuerle and Baltimore 1988). In addition, a variety of proteases are expressed in the skin. These proteases are involved in

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maintaining homeostasis through immune response, inflammation, cytokine expression, vascular function, tissue recovery, host defense, and apoptosis (Sharlow et al. 2000).

Protease-activated receptor 2 (PAR2) plays an important role in inflammation. In 1995, we observed PAR-2 expression in Santulli in vitro. D 'Andrea observed PAR-2 expression and formed surface, superficial, and granular layers. PAR-2 and PAR-4 indicate that trypsin activates mast cell tryptase PAR-2 (Santulli et al. 1995; Coughlin 1999; D'Andrea et al. 1998). In addition, trypsin, a mast cell tryptase or SLGRL-NH2 (synthetic peptide) also activates PAR-2 and causes extensive inflammation (Kong et al. 1997).

Prunin is a flavonoid, which is a family of polyphenolic compounds with its name derived from the Greek word flavus that means yellow. This plant pigment possesses flavone as its basic structure. Found in relatively small amounts in animals, prunin is mainly present in vegetables, fruits, flowers, leaves, stems, roots, and fruits (Middleton et al. 2000). Prunin is a flavanone glycoside with the molecular formula $C_{21}H_{22}O_{10}$ and a molecular weight of 434.39. Prunin was isolated from *P. davidiana* Fr. (Rosacea) stems (Jung et al. 2017). The stems of *P. davidiana* Fr. (Rosacea) are used in Korean traditional medicine for treating neuritis and rheumatism (Choi et al. 1991). These stems contain antiviral agents and a variety of flavonoids that possess antioxidant, anti-inflammatory, hypocholesterolemic, and cardioprotective properties, and the DNA-binding activity (Jung et al. 2003; Yousuf et al. 2013; Zhang et al. 2008). Previous studies on prunin report that it has antioxidant and anti-inflammation properties, decreases blood glucose and insulin levels, and improves blood circulation (Céliz et al. 2013; Céliz et al. 2010; Céliz et al. 2011; Choi et al. 2006; Kimihisa et al. 2010). In this study, we investigated the effects of prunin treatment on ultraviolet (UVB)-induced cell damage and inflammation.

Methods
Cell culture
The human keratinocyte HaCaT cells used in this experiment were purchased from ATCC (USA). To incubate HaCaT cell strains, Dulbecco's Modified Eagle Medium (Hyclone, USA) containing 10% fetal bovine serum (Hyclone) and 1% penicillin/streptomycin (penicillin 100 IU/ml, streptomycin 100 μg/ml; Invitrogen, USA) was used. The cells were incubated at 37 °C with 5% CO_2.

Sample treatment
Prunin was purchased from Sigma-Aldrich (USA) in purified (> 95%) powder, which was dissolved in

dimethyl sulfoxide (Sigma-Aldrich) at an appropriate concentration for the experiment. HaCaT (1×10^6cell/well) was incubated in the culture dish for 24 h, and prunin was subsequently added at an appropriate concentration and preprocessed for 6 additional hours. A UVB lamp (UVP, USA) was used to irradiate the cells with UVB. The wavelength of UVB irradiation was measured with a fiberoptic Spectrometer System USB2000 (Ocean Optics, USA). After the irradiation, PBS was removed, and a new medium was added, followed by additional incubation of 24 h prior to use in the experiment.

Cell viability estimation
The principles of the WST-1 assay were used for the experiment on cell survival. Overall, 100 μL HaCaT (3×10^3cells/well) was inoculated on a 96-well plate and incubated for 24 h. Afterward, prunin was added at 1, 5, 10, 20, and 40 μM and 40 mJ/cm^2 and UVB was irradiated to these cells, which were subsequently incubated for additional 24 h. In addition, 10 μL of EZ-Cytox cell viability assay kit reagent (ITSBio, Korea) was added to the incubated cells for 1 h, and the absorbance was measured using a microplate reader (Bio-Rad, USA) at 490 nm.

Quantitative analysis of intracellular ROS
In total, 10 μM dichlorofluorescein diacetate was added as a dye to measure intracellular ROS. The cells were harvested after incubation for 30 min. After adding PBS to release the cells, a flow cytometer (BD Biosciences, USA) was used to measure the changes in ROS. To verify the removal effects of prunin on ROS, N-acetyl-L-cysteine (NAC) was used as a positive control group that acts as a ROS scavenger and measured after the same treatment as prunin.

Quantitative real-time PCR
The cells obtained from the cell culture were extracted with Trizol reagent (Invitrogen, USA). The extracted RNA was quantified using a Nanodrop (Maestrogen, USA), and only RNA with a purity of 260 nm/280 nm (ratio 1.8) was used for the experiment.

In total, 10 μl of DNA was prepared by adding 1 μg RNA, 0.5 ng oligo dT18, and DEPC water in a PCR tube and processing it for 10 min at 70 °C to induce RNA denaturation. Subsequently, M-MLV reverse transcriptase (Enzynomics, Korea) was used to induce a reaction at 37 °C for 1 h to synthesize cDNA.

For qRT-PCR, 0.2 μM primers, 50 mM KCl, 20 mM Tris/HCl pH 8.4, 0.8 mM dNTP, 0.5 U Extaq DNA polymerase, 3 MgCl2, and 1X SYBR green (Invitrogen) were mixed in a PCR tube to produce a reagent. PCR was performed with Linegene K (BioER, China) through

Table 1 List of primers used in this study

Gene	Forward primer	Reverse primer
β-actin	GGATTCCTATGTGGGCGACGA	CGCTCGGTGAGGATCTTCATG
TNF-α	CCCAGGGACCTCTCTCTAATC	GGTTTGCTACAACATGGGCTACA
IL-6	TAACAGTTCCTGCATGGGCGGC	AGGACAGGCACAAACACGCACC
IL-8	CTCTCTTGGCAGCCTTCC	CTC AATCACTCTCAGTTCTTTG
COX2	CGCGGATCCGCGGTGAGAAC CGTTTAC	GCGAGGAAGCGGAAGAGTCT AGAGTCGACC
PAR-2	GGGTTTGCCAAGTAACGGC	GGGAACCAGATGACAGAGAGG

40 cycles (3 min per cycle) of denaturation, denaturation (94 °C, 30 s), annealing (58 °C, 30 s), and polymerization (72 °C, 30 s) at 94 °C. The significance of PCR was validated using a melting curve. The gene expressions were compared for analysis by normalizing the β-actin expression. The primers used for this experiment are shown in Table 1.

Statistical analysis
Each experiment in this study was performed three times or more under the same conditions, and the results are expressed as mean and standard deviation. For each experiment, the Student's *t* test was used to analyze all findings, with a *p* value of 0.05 or below considered as statistically significant.

Results
Cytotoxic and protective effects of prunin
The water-soluble tetrazolium salt-1 assay was performed to determine the cytotoxicity of prunin in human keratinocyte HaCaT cells. Treatment of the cells with prunin at concentrations of 1, 5, 10, 20, and 40 μM revealed the following survival rates: 1 μM, 100%; 5 μM, 102%; 10 μM, 102%; 20 μM 93%, and 40 μM, 75%. These results suggest that prunin is cytotoxic at concentrations ≥ 40 μM (Fig. 1a). To examine the cytoprotective effects of prunin, damaged HaCaT cells were treated with
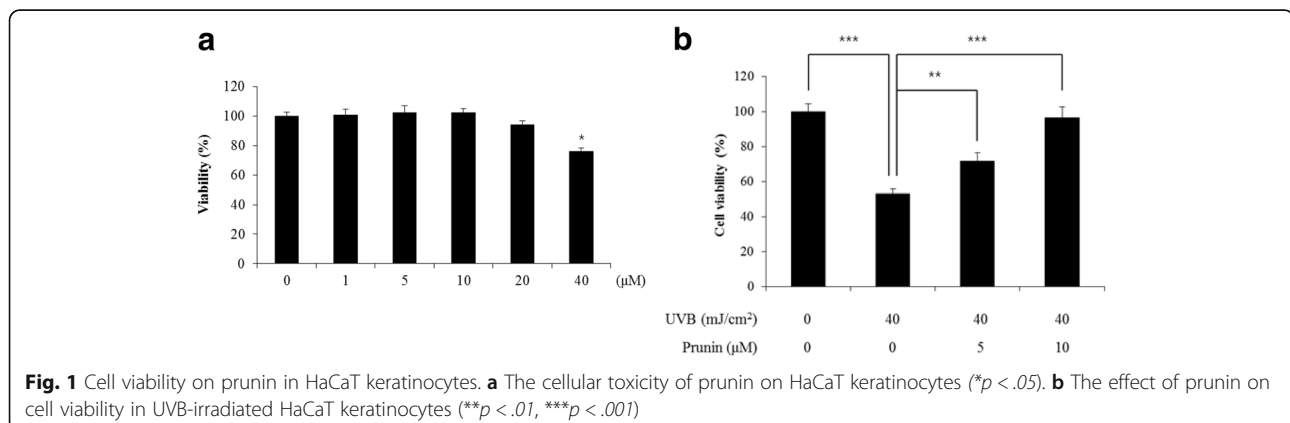
prunin (5 or 10 μM) and irradiated with 40 mJ/cm^2 UVB. Compared to the cell survival rate of 53% in the control group, the survival rate of prunin-treated cells increased to 71% (5 μM) and 96% (10 μM). The cell survival rate was increased by prunin treatment in a concentration-dependent manner (Fig. 1b).
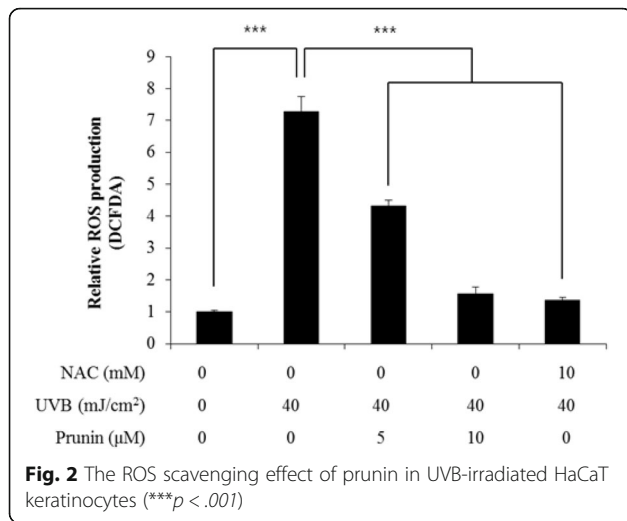
ROS elimination effect of prunin in HaCaT cells irradiated with UVB
The cell-permeable reagent 2′,7′-dichlorofluorescin diacetate was used to investigate the effect of prunin on ROS in UVB-irradiated HaCaT cells. This reagent fluorescent stains intracellular ROS, which were then analyzed by flow cytometry. ROS increased from 1 to 7.28 in cells exposed to 40 mJ/cm^2 UVB. Treatment of cells with prunin at concentrations of 5 and 10 μM before irradiation resulted in a decrease in ROS to 4.32 and 1.57, respectively. As a positive control, treatment with 10 mM NAC resulted in a decrease to 1.35, a result similar to that after treatment with 10 μM prunin (Fig. 2).

Anti-inflammatory effect of prunin on UVB-irradiated HaCaT cells
The expression level of IL-6, IL-8, COX2, TNF-α, and PAR2 was confirmed by quantitative real-time PCR to assess the effect of prunin on UVB-induced inflammation in HaCaT cells. First, we examined the effect of prunin on the UVB-induced increase in *IL-6* mRNA (5.17). Cells treated with prunin at concentrations of 5 and 10 μM before UVB exposure exhibited a decrease in *IL-6* mRNA to 3.55 and 1.40, respectively. Prunin treatment decreased *IL-6* mRNA expression in a concentration-dependent manner (Fig. 3a). The *IL-8* mRNA expression of 3.17 after UVB exposure decreased to 2.43 and 1.78 with prunin treatment at 5 and 10 μM, respectively. Prunin decreased *IL-8* mRNA expression in a concentration-dependent manner (Fig. 3b). *COX2* mRNA, encoding a protein that induces inflammation,
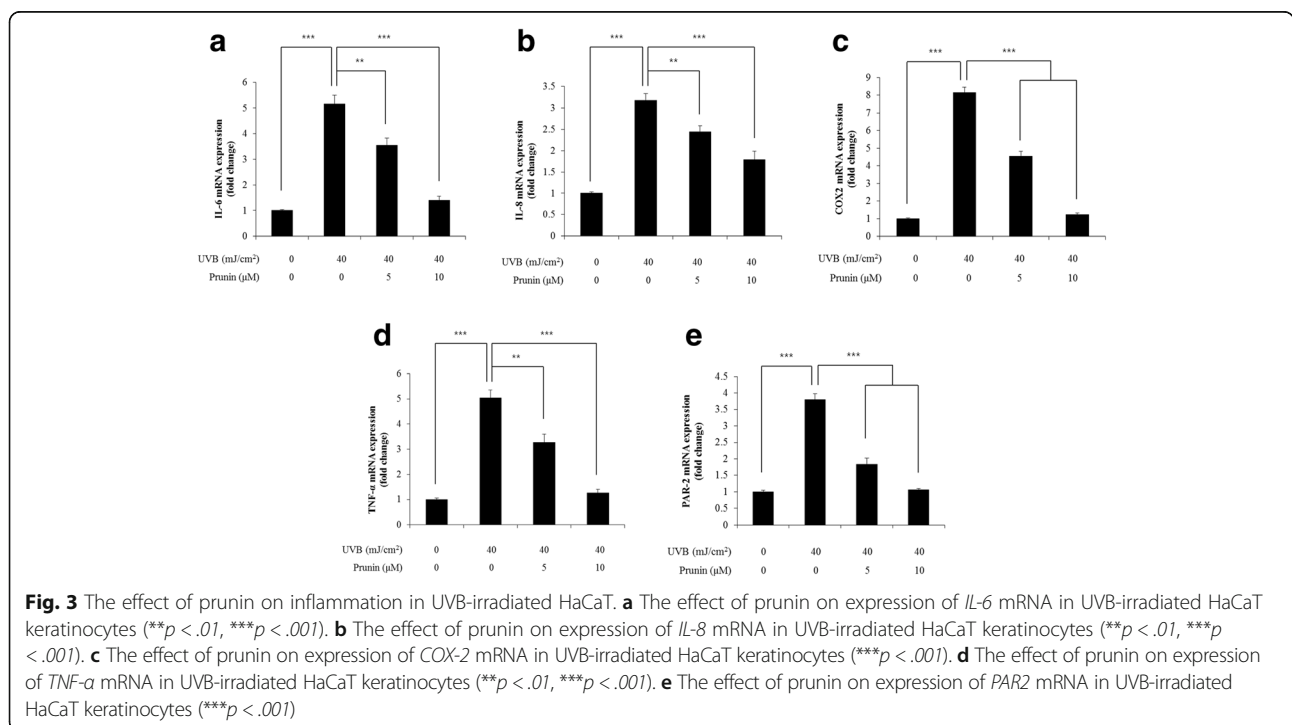

Fig. 1 Cell viability on prunin in HaCaT keratinocytes. **a** The cellular toxicity of prunin on HaCaT keratinocytes (*p < .05). **b** The effect of prunin on cell viability in UVB-irradiated HaCaT keratinocytes (**p < .01, ***p < .001)

Fig. 2 The ROS scavenging effect of prunin in UVB-irradiated HaCaT keratinocytes (***$p < .001$)

increased to 8.14 after UVB exposure. Pretreatment with 5 or 10 μM prunin decreased *COX2* mRNA expression to 4.55 and 1.23, respectively. Prunin decreased *COX2* mRNA expression in a concentration-dependent manner (Fig. 3c). *TNF-α* mRNA expression increased to 5.04 after UVB exposure. Pretreatment with 5 or 10 μM prunin decreased *TNF-α* mRNA expression to 3.26 and 1.26, respectively. Prunin decreased *TNF-α* mRNA expression in a concentration-dependent manner (Fig. 3d). *PAR2* mRNA increased to 3.80 upon UVB exposure.

Pretreatment with 5 or 10 μM prunin resulted in a decrease in *PAR2* mRNA expression to 1.83 and 1.06, respectively. Prunin decreased *PAR2* mRNA expression in a concentration-dependent manner (Fig. 3e).

Discussion

Intracellular damage by oxidative stress causes cell cycle arrest and cellular aging and increases the production of ROS (Cerella et al. 2009). Intracellular ROS can damage DNA and mitochondria, resulting in abnormalities in energy metabolism along with protein oxidation (Lang et al. 2018). In addition, ROS activates NF-κB, which promotes the expression of genes that induce inflammatory responses (Nathan 1987; Baeuerle and Baltimore 1988).

We observed here that exposing HaCaT cells to 40 mJ/cm^2 UVB leads to an increase in ROS. Figure 2 shows that prunin inhibits this UVB-induced increase in ROS in HaCaT cells in a concentration-dependent manner, inhibits the increased cellular aging caused by UVB, and is effective for ROS elimination. In addition, we observed that exposure of these cells to 40 mJ/cm^2 UVB causes a rapid increase in the activity of NF-κB, a proinflammatory transcription factor. Prunin pretreatment inhibited this increase in activity in a concentration-dependent manner. In other words, prunin inhibits the activation of NF-κB in the nucleus, thereby decreasing the expression of several inflammation-related substances. In particular, the expression levels of *IL-8*, *IL-6*, *COX2*, *TNF-α*, and

Fig. 3 The effect of prunin on inflammation in UVB-irradiated HaCaT. **a** The effect of prunin on expression of *IL-6* mRNA in UVB-irradiated HaCaT keratinocytes (**$p < .01$, ***$p < .001$). **b** The effect of prunin on expression of *IL-8* mRNA in UVB-irradiated HaCaT keratinocytes (**$p < .01$, ***$p < .001$). **c** The effect of prunin on expression of *COX-2* mRNA in UVB-irradiated HaCaT keratinocytes (***$p < .001$). **d** The effect of prunin on expression of *TNF-α* mRNA in UVB-irradiated HaCaT keratinocytes (**$p < .01$, ***$p < .001$). **e** The effect of prunin on expression of *PAR2* mRNA in UVB-irradiated HaCaT keratinocytes (***$p < .001$)

PAR2, which are induced upon activation of NF-κB, decreased with increasing concentrations of prunin. These results suggest that prunin inhibits UVB-induced inflammation in HaCaT cells and therefore is effective for anti-inflammatory skin.

Conclusions

Prunin pretreatment protects HaCaT cells against UVB-induced damage by preventing the generation of ROS and decreasing the expression of *IL-6*, *IL-8*, *COX2*, *TNF-α*, and *PAR2* in a concentration-dependent manner. Prunin improves the survival rate of UVB-damaged HaCaT cells and blocks the NF-κB pathway through ROS elimination, thereby inhibiting inflammatory responses. In summary, prunin has cytoprotective and anti-inflammatory effects in HaCaT cells.

Abbreviations
11β-HSD1: 11β-hydroxysteroid ehydrogenase type 1; COX2: Cyclooxygenase-2; IL-6: Interleukin-6; IL-8: Interleukin-8; IκB: Inhibitor kappa B; NAC: N-acetyl-L-cysteine; NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells; PAR2: Protease-activated receptor 2; ROS: Reactive oxygen species; TNF-α: Tumor necrosis factor-alpha; UVB: Ultraviolet

Availability of data and materials
Not applicable

Authors' contributions
EJN and JYR did all of research background such as experiments, data collecting, and statistical analysis as well as draft manuscript. Both authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests.

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