Hyperin and Quercetin Modulate Oxidative Stress-Induced Melanogenesis

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Hyperin and quercetin are phenolic compounds present in fruits and vegetables that have been reported to possess strong anti-oxidative properties. Although increasing evidence strongly suggests that antioxidants suppress the melanin synthesis that is causally associated with oxidative stress, the protective actions of hyperin and quercetin against oxidative stress-induced melanogenesis have not been fully explored. To elucidate the suppressive effects of hyperin and quercetin on oxidative stress and melanin synthesis, peroxynitrite (ONOO[−]) scavenging activity was measured *in vitro* as were quantifications of melanin content, intracellular total RS, ONOO[−], superoxide (O₂^{•−}), nitric oxide (NO[•]), catalase activity and the reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio. Results showed that *in vitro*, hyperin and quercetin reduced ONOO[−]. Additionally, hyperin and quercetin suppressed total RS, ONOO[−], O₂^{•−}, and NO[•], catalase activity, and melanin synthesis, while they boosted the GSH/GSSG ratio in B16F10 melanoma cells (B16 cells). Therefore, I propose that hyperin and quercetin have a powerful capacity to modulate oxidative stress-induced melanogenesis.

Key words hyperin; quercetin; antioxidant; oxidative stress; melanogenesis

Melanogenesis is the process by which pigment forms and melanin is synthesized in special organelles called melanosomes.¹⁾ Melanin plays a key role in the protection of the skin against deleterious sunlight under normal conditions, but also can increase the generation and excessive accumulation of melanin that lead to clinical and cosmetic concerns such as freckles, age spots, sites of actinic damage, and melasma. Chronic inflammation, UV ray radiation, and the release of abnormal α -melanocyte stimulating hormone (α -MSH) also are well-known triggering factors for abnormal melanogenesis and inflammatory pigmentation.^{1,2)}

Oxidative stress caused by excessive reactive species (RS), including reactive oxygen species (ROS) and reactive nitrogen species (RNS), is known to be associated with skin aging and hyperpigmentation. Also, a deficient anti-oxidative defense system, such as a disturbed redox balance and weak anti-oxidant enzyme activity, exacerbate skin aging and hyperpigmentation.^{3–5)} Therefore, searching for natural antioxidants that inhibit melanogenesis has been an important strategy in the development of skin medications and cosmetics.⁶⁾ We have reported on the significance of maintaining cellular redox through the down-regulation of RS and the boosting of anti-oxidant defenses in the inhibition of melanogenesis.^{5,7,8)}

Various phenolic compounds from natural sources have been reported as melanogenic inhibitors.^{5,7–11)} Hyperin and quercetin are two phenolic flavonoids reported to have anti-oxidative¹²⁾ and anti-viral¹³⁾ properties. However, the effects of hyperin and quercetin on oxidative stress-induced melanogenesis have not been fully explored to date.

To estimate the effects of hyperin and quercetin on oxidative insult *in vitro*, peroxynitrite (ONOO[−]) scavenging activity was measured. In addition, we used B16F10 melanoma cells (B16 cells) to investigate the effect of hyperin and quercetin on melanin content and performed measurements of several key oxidative and anti-oxidative markers: total RS generation, ONOO[−], superoxide (O₂^{•−}), nitric oxide (NO[•]), reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio, and

antioxidant enzyme (catalase) activity.

MATERIALS AND METHODS

Reagents L-Tyrosine, α -MSH, and other chemical reagents were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell Lines and Cultures B16 cells (from Korean Cell Line Bank, Korea) were maintained in Dulbecco's modified Eagle's medium (DMEM) medium supplemented with 10% fetal bovine serum (FBS, Gibco, NY, U.S.A.) containing penicillin/streptomycin (100 IU/50 g/mL), at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. These B16 cells were cultured in 24-well plates for each assay. All the experiments were performed in triplicate and repeated three times to ensure reproducibility.

Determination of Cell Viability The cell viability assay was carried out as described¹⁴⁾ using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co.). Shortly thereafter, 5×10^4 cells/well were plated in a 24-well plate. After cells were exposed separately to hyperin and quercetin at concentrations of 5–100 μ M for 24 h, MTT solutions were added and the insoluble derivative formed by cellular dehydrogenase was solubilized with EtOH–dimethyl sulfoxide (DMSO) (1:1 mixture solution); the absorbance of each well was estimated at 560 nm using a microplate reader.

ONOO[−] Levels *in Vitro* ONOO[−] scavenging was measured by monitoring the oxidation of DHR 123 by the method of Kooy *et al.*¹⁵⁾ We added samples to the rhodamine buffer (pH 7.4) containing 6.25 μ M DHR 123 and 125 μ M DTPA and incubated the mixture for 5 min at 37°C. Absorbance was measured at 500 nm, which is the absorbance of rhodamine 123.

The Effect of Hyperin and Quercetin on Total RS Generation in B16 Cells Total RS production was measured in culture supernatant.¹⁶⁾ Twenty-five millimolar 2',7'-dichlorofluorescein diacetate (DCFH-DA) was added to the incubation media, and changes in fluorescence were determined at an

excitation wavelength of 486nm and emission wavelength of 530nm for 30min.

Measurement of NO[•] Generation in B16 Cells NO[•] generation was examined by measuring the accumulation of nitrite in the conditioned medium by the Griess assay. Briefly, 100 μ L of culture supernatant was allowed to react with 100 μ L of the Griess reagent¹⁷⁾ and then incubated at room temperature for 5 min. The optical density of the samples at 540 nm was read using a microplate reader.

Evaluation of [•]O₂ Levels in B16 Cells [•]O₂ levels were estimated following the method as previously described.¹⁸⁾ [•]O₂-scavenging activity was evaluated by assessing the decrease in the ratio of the reduction of nitro blue tetrazolium (NBT). The culture supernatant was added to the reaction buffer (50 mM phosphate buffered saline (PBS) with 125 μ M ethylenediaminetetraacetic acid (EDTA), 62 μ M NBT and 98 μ M reduced nicotinamide adenine dinucleotide) containing 33 μ M 5-methylphenazium methyl sulfate. The absorbance at 540 nm, as an index of NBT reduction, was estimated after 5 min.

Determination of ONOO⁻ in B16 Cells ONOO⁻-dependent oxidation of dihydrorhodamine 123 (DHR 123) to rhodamine 123 was evaluated based on the method as described previously.¹⁵⁾ Samples were added to the rhodamine buffer (pH 7.4) containing 6.25 μ M DHR 123 and 125 μ M diethylenetriaminepentaacetic acid (DTPA) and incubated 5 min at 37°C. Absorbance was measured at 500 nm, which is the absorbance of rhodamine 123.

Measurement of Catalase Activity in B16 Cells Catalase activity was estimated spectrophotometrically by direct measurement in the reduction of light absorption at 240 nm caused by the decomposition of hydrogen peroxide by catalase.¹⁹⁾

Estimation of GSH and GSSG Levels in B16 Cells GSH levels were assayed according to the method of Pandey and Katiyar.²⁰⁾ Twenty-five percent of the *meta*-phosphoric acid-added cell pellets were centrifuged at 12000 $\times g$ for 10 min, and then the supernatant was taken for assay. One millimolar EDTA–50 mM phosphate buffer was added to the supernatant followed by *o*-phthalaldehyde. After 20 min at room temperature, the fluorescence was measured at excitation wavelength of 360 nm and emission wavelength of 460 nm. GSSG levels were determined after preincubation with *N*-ethylmaleimide for 20 min, and 0.1 M NaOH was replaced for 1 mM EDTA–50 mM phosphate buffer.

Determination of Melanogenesis in B16 Cells In this present study, the amount of melanin content was used as an index of melanogenesis. An assay to measure the amount of melanin content followed a modified method of Bilodeau *et al.*²¹⁾ Briefly, B16 cells (5 $\times 10^4$) were plated on 24-well, multi-dishes and incubated in the presence or absence of 100 nM α -MSH. Cells were then incubated for 24 h with or without hyperin and quercetin at concentrations ranging from 5 to 50 μ M. After washing twice with PBS, samples were dissolved in 100 μ L of 1 N NaOH. The samples were incubated at 60°C for 1 h and mixed to solubilize the melanin. Absorbance was measured at 405 nm and compared with a standard curve of synthetic melanin.

Protein Assay The concentration of protein was examined by a bicinchoninic acid protein assay.²²⁾ All samples were assayed in triplicate.

Statistical Analysis Data were analyzed as mean $\pm$ standard error ($n=5$) and the biological significance $p<0.05$ was

Fig. 1. Chemical Structures of Hyperin and Quercetin

Fig. 2. The Effect of Hyperin and Quercetin on Cell Viability

Cells treated with various doses of the hyperin and quercetin were evaluated by the MTT assay. Data are expressed as % of cell viability.

determined by the Student's *t*-test.

RESULTS AND DISCUSSION

This study was designed to determine the effects of hyperin and quercetin on oxidative stress-induced melanogenesis. To the best of my knowledge, this present study shows the first evidence that both hyperin and quercetin have a strong suppressive effect on oxidative stress-related melanogenesis in B16 cells.

It has been reported that hyperin and quercetin possess antioxidant properties.²³⁾ Their structural analysis (see the structures of hyperin and quercetin in Fig. 1) reveals that the unique difference between quercetin and hyperin lies in position 3 of ring C, where a hydroxyl group and galactose exist, respectively.²⁴⁾ The hydroxyl group on position 3 in ring C is associated with hydroxyl radical-scavenging activity. Therefore, the assumption is that quercetin would show more potent hydroxyl radical scavenging activity than hyperin²⁴⁾ and would therefore bear out our results that quercetin showed stronger antioxidant activity than hyperin due to their structural differences. Findings in the current study also correlate with previous reports indicating that aglycone exhibits a more potent apoptotic property than the corresponding glycosylated compound,²⁵⁾ and TPA-induced prostaglandin E₂ (PGE₂) production in rat peritoneal macrophages.²⁶⁾

The potential use of hyperin and quercetin as skin-lightening agents also was evaluated by measuring cell cytotoxicity. As displayed in Fig. 2, hyperin and quercetin were found to

Table 1. Effects of Hyperin and Quercetin on ONOO⁻ Scavenging Activity *in Vitro*

Compound	IC ₅₀ (μM)
Hyperin	0.89±0.14
Quercetin	0.78±0.05
Penicillamine	3.01±0.47

Table 2. Effects of Hyperin and Quercetin on Melanogenesis

Group	Melanin content (%)
Control	11.63±0.32
Hyperin (5 μM)	8.23±1.20**
Hyperin (10 μM)	6.22±0.92**
Hyperin (50 μM)	4.12±0.23***
Quercetin (5 μM)	3.12±0.03*
Quercetin (10 μM)	2.01±0.91*
Quercetin (50 μM)	1.12±0.33**

Each value represents the mean±S.E.M. of three determinations. Statistically significant differences in comparison with control group (**p*<0.05, ***p*<0.01, ****p*<0.001).

Table 3. Effects of Hyperin and Quercetin on Total RS Production

Group	Total RS production (fluorescence/min/mg protein)
Control	9.62±0.46
Hyperin (5 μM)	8.01±1.21**
Hyperin (10 μM)	7.34±1.01**
Hyperin (50 μM)	5.83±0.32***
Quercetin (5 μM)	2.21±0.67*
Quercetin (10 μM)	1.53±0.87*
Quercetin (50 μM)	0.78±0.29**

Each value represents the mean±S.E.M. of three determinations. Statistically significant differences in comparison with control group (**p*<0.05, ***p*<0.01, ****p*<0.001).

have no significant cytotoxic effects at any of the concentrations tested. Because oxidative stressors and RS are closely associated with melanogenesis,³⁻⁵) and melanin synthesis is enhanced under increased oxidative stress that generally leads to hyperpigmentation, the anti-oxidative action of hyperin and quercetin was then measured (Table 1).

We next sought to experimentally substantiate the suppression of melanogenesis in B16 cells by hyperin and quercetin; the results are shown in Table 2. Additionally, our results showing the efficacy of hyperin and quercetin on various other oxidative species are displayed in Table 3. Both hyperin and quercetin effectively inhibited total RS generation, implying the possibility that a number of hydroxyl groups of hyperin and quercetin that are likely responsible for the suppression of total RS, which is consistent with previous studies.^{5,11,23})

Data shown in Figs. 3–5 further support the notion that that [•]O₂, NO[•], and ONOO⁻ production were blunted by hyperin and quercetin. Interestingly, both hyperin and quercetin showed strong ONOO⁻ scavenging activity both *in vitro* and in B16 cells, indicating that controlling ONOO⁻ is one of the major contributing factors in melanogenesis inhibition by hyperin and quercetin. This fact is supported by the previous study²⁷⁾ showing that the ONOO⁻ scavenging property is a crucial factor in melanogenic regulation. In addition,

Fig. 3. Effect of the Hyperin and Quercetin on [•]O₂ Levels

Each value represents the mean±S.E.M. of at least three determinations that are significantly different from control group (***p*<0.01, ****p*<0.001).

Fig. 4. Suppressive Action of Hyperin and Quercetin on NO[•] Levels

Results are expressed as the mean±S.E.M. of at least three determinations. Statistically significant differences from the control group are noted as ****p*<0.001.

Fig. 5. The Inhibitory Property of Hyperin and Quercetin on ONOO⁻

Results are expressed as the mean±S.E.M. of at least three determinations. Statistically significant differences from the control group are noted as **p*<0.05, ***p*<0.01, ****p*<0.001.

Fig. 6. The Augmenting Effect of Hyperin and Quercetin on Catalase Activity

Each value represents the mean±S.E.M. of at least three determinations that are significantly different from the control group (** $p<0.01$, *** $p<0.001$).

we recently reported that reducing total RS, $\text{O}_2^{\cdot-}$, $\text{NO}^{\cdot}$, and ONOO^- may well be major underlying factors in the suppression of melanogenesis.⁸⁾

One of the novel findings of the current study is the control of catalase activity by hyperin and quercetin. Figure 6 reveals that hyperin and quercetin modulated catalase activity, indicating that in B16 cells, the modulation of catalase by hyperin and quercetin correlates with regulation of melanogenesis.²⁸⁾

It was recently reported that the disturbance of RS regulation results in redox imbalance, causing oxidative insult.^{29,30)} Figures 7A–C show that hyperin and quercetin enhanced GSH levels and the GSH/GSSG ratio while suppressing GSSG levels, indicating the ability of hyperin and quercetin to accentuate the cellular redox status in B16 cells. These data imply that sustaining redox balance by hyperin and quercetin also might be an important contributing factor for melanogenesis suppression, which is consistent with recent reports.^{31,32)}

Thus, the major findings of our present work are that hyperin and quercetin exert a broad and potent anti-oxidative efficacy against total RS, $\text{O}_2^{\cdot-}$, $\text{NO}^{\cdot}$, and ONOO^- and enhance the GSH/GSSG ratio and catalase activity in B16 cells. The present study demonstrated that hyperin and quercetin modulate oxidative stress-induced melanogenesis not only by suppressing total RS, $\text{O}_2^{\cdot-}$, $\text{NO}^{\cdot}$, ONOO^- , but also by boosting the anti-oxidative ONOO^- scavenging capacity, the GSH/GSSG ratio and catalase activity in B16 cells. In conclusion, this present work strongly indicates that hyperin and quercetin can be possible potent oxidative stress-induced melanogenic inhibitors. Based on these findings, we postulate that the amelioration of oxidative stress might be the plausible mechanism underlying the inhibition of melanogenesis by hyperin and quercetin, which, in turn, may contribute to the development of new depigmenting agents. However, more molecular and clinical investigations are warranted to elucidate the precise biological mechanisms of hyperin and quercetin.

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Fig. 7. The Effect of Hyperin and Quercetin on (A) GSH Level (B) GSSG Level and (C) GSH/GSSG Ratio

Each value represents the mean±S.E.M. of at least three determinations that are significantly different from the control group (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

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