Modulation of Oxidative Stress and Melanogenesis by Proanthocyanidins

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Proanthocyanidins (PAs) are polymer chains of flavonoids known to have a high free radical scavenging capacity. However, their efficacy for use in dermatological health has not been fully explored. In the present study, we investigated the inhibitory property of PAs on melanogenesis and oxidative stress of cultured B16F10 melanoma cells (B16 cells) utilizing both oligomer and polymer PAs that were isolated from freshly crushed persimmon peel. To assess the suppressive effects of PAs against oxidative insults, lipid peroxidation, total reactive species (RS), peroxynitrite (ONOO$^-$), superoxide (O$_2^-$), and nitric oxide (NO$^+$) were quantitated. In addition, the reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio was measured to evaluate the cellular oxidative status. Results showed that the PAs studied had a strong inhibitory effect on the murine tyrosinase and melanin synthesis that was correlated with the modulation of oxidative stress. Thus, our present work produced evidence that in B16 cells, the anti-melanogenic capacity of PAs as shown by the inhibition of tyrosinase and melanin synthesis likely occurs through the suppression of oxidative stress by the ability of PAs to modulate total RS, O$_2^-$, NO$^+$, ONOO$^-$, lipid peroxidation, and redox balance.

Key words proanthocyanidin; flavonoid; melanogenesis; oxidative stress; redox balance

MATERIALS AND METHODS

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

Isolation and Preparation of Persimmon Peel PAs As described previously in detail,\textsuperscript{20} a mixture of freshly crushed persimmon peel (green peel 5—7 cm in diameter, 3 kg) and dried green tea leaves (450 g) in water containing citric acid (240 g) was boiled for 3 h. At this stage, nucleophilic substitution at the C-4 positions of polymeric PA with monomeric tea catechins occurred, and as a result, the polymeric molecules were converted into oligomers. After cooling, the insoluble materials were removed by filtration, and the filtrate was directly applied to a Sephabeads SP 825 column (10 cm i.d.$\times$45 cm, Mitsubishi Chemical Co., Tokyo, Japan). Elution with water (4 l) washed out the non-phenolic compounds consisting of citric acid, sugars, minerals, amino acids, etc. Further elution with water containing augmenting amounts of ethanol (20—80% ethanol, 20% stepwise elution, each 2 l) yielded a mixture of oligomeric PA and tea catechins (72.2 g). The mixture was subsequently subjected to Sephadex LH-20 column chromatography with ethanol elution to isolate the monomeric tea catechins, and further elution with 50% aqueous acetone yielded an oligomer

Melanin is responsible for skin pigmentation, and is synthesized in special organelles called melanosomes\textsuperscript{1} that play an essential role in the protection of the skin from deleterious sunlight under normal conditions. However, enhanced generation and excessive accumulation of melanin can cause a number of skin problems such as freckles, age spots, and melanoma. UV ray radiation, chronic inflammation, and abnormal $\alpha$-melanocyte stimulating hormone ($\alpha$-MSH) release are well-known triggering factors for abnormal melanin synthesis and inflammatory pigmentation.\textsuperscript{2} Therefore, the inhibitors of the melanogenesis, particularly from natural sources, have been of great interest to industries that produce skin medications and cosmetics.\textsuperscript{1}

The enzymatic action of tyrosinase is a key step in melanogenesis as it catalyzes the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and L-DOPA to dopaquinone.\textsuperscript{3} Thus, many tyrosinase inhibitors from both synthetic and natural sources have been studied: kojic acid, hydroquinone,\textsuperscript{3} 3,4-dihydroxyacetophenone,\textsuperscript{4} and 4,4'-dihydroxybiphenyl.\textsuperscript{5}

Skin is incessantly exposed to both endogenous and exogenous oxidative stress and the resultant oxidative damages are induced by excessive reactive species (RS) and lipid peroxidation. RS are known to play a major role in the formation of skin pigmentation and the cause of skin aging.\textsuperscript{5—8}

Data from ours\textsuperscript{9—12} and those of Imokawa\textsuperscript{13} have shown the importance of maintaining cellular redox by the suppression of RS, and boosting anti-oxidative defenses for anti-melanogenic activity.

A number of naturally occurring polyphenolic compounds have anti-oxidant activity,\textsuperscript{14} and many of them show anti-melanogenic activity containing a phenolic structure. Recently, we\textsuperscript{9—12,15} and others\textsuperscript{16,17} have reported on many polyphenolic compounds from natural sources as melanogenic inhibitors. Proanthocyanidins (PAs) from persimmon (Diospyros kaki) peel have polyphenolic compound properties that are reported to have anti-oxidative, anti-fungal and anti-cancer actions.\textsuperscript{18,19} However, the effect of PAs from persimmon peel on melanogenesis has not been explored to date. The biological activities of PAs may be related to the extent of their polymerization,\textsuperscript{19} but a correlation between the effects and degree of polymerization has not been made clear. In the current study, we used B16F10 melanoma cells (B16 cells) to investigate and compare the inhibitory property of both the oligomer and polymer forms of PAs on several key oxidative and anti-oxidative markers: thiobarbituric acid reactive substances (TBARS), RS generation, peroxynitrite (ONOO$^-$), superoxide (O$_2^-$), nitric oxide (NO$^+$), and the reduced glutathione (GSH)/oxidized glutathione (GSSG) ratio.

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(51.5 g). The degree of oligomeric polymerization was estimated as 3.3 by quantitative HPLC analysis of thiol degradation products, while the unit ratios of epigallocatechin, epicatechin, epigallocatechin 3-O-gallate, and epicatechin 3-O-gallate in oligomer were determined as 47, 15, 31, and 6%, respectively. The preparation of persimmon polymeric PA was as follows: an aqueous acetone extract of fresh persimmon peel (500 g) was concentrated, and the resulting insoluble precipitates were removed by filtration. The filtrate was subjected to MCI-gel CHP 20P (Mitsubishi Chemicals Co., Tokyo, Japan) column chromatography with water containing methanol (0—80%, 20% stepwise elution) to give polymer (6.93 g).

Procedure for B16 Cell Culture B16 cells (from the Riken Cell Bank, Tsukuba, Japan) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS, Gibco, NY, U.S.A.) and penicillin/streptomycin (100 IU/50 μg/ml) in a humidified atmosphere containing 5% CO₂ in air at 37 °C. These B16 cells were cultured in 24-well plates for each assay. All the experiments were carried out in triplicate and repeated three times to ensure reproducibility.

Cell Viability The cell viability assay was performed as described using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO, U.S.A.). Shortly thereafter, 5×10⁴ cells/well were plated in a 24-well plate. After 24 h, cells were exposed to PA at concentrations of 5—50 μg/ml 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 read at 560 nm using a microplate reader.

The Effect of PAs on Melanogenesis and Oxidative Stress in B16 Cells. a) Assay for Murine Tyrosinase Activity Tyrosinase activity in B16 cells was determined by measuring the rate of oxidation of L-DOPA. Cells were plated in a 24-well plate. After cells were exposed to PA at concentrations ranging from 10 to 400 μM, the absorbance at 492 nm was read every 10 min for 1 h at 37 °C using a microplate reader.

b) Evaluation of Melanin Synthesis in B16 Cells In this work, the amount of melanin content was used as an index of melanogenesis. An assay measuring the amount of melanin content was performed using a modified method of Bilodeau et al. Briefly, B16 cells (5×10⁴) were plated on 24-well, multi-dishes and incubated in the presence or absence of 100 ng α-MSH. Cells were then incubated for 24 h with or without PA at concentrations ranging from 10 to 400 μM. After washing twice with phosphate-buffered saline (PBS), samples were dissolved in 100 μl of 1 N NaOH. The samples were incubated at 60 °C for 1 h and mixed to solubilize the melanin. Absorbance at 405 nm was compared with a standard curve of synthetic melanin.

Investigations of the Inhibitory Effects of PA against Oxidative Stress in B16 Cells. a) Measurement of Lipid Peroxidation by TBARS The concentration of TBARS was determined according to Buege and Aust with slight modification. This method involves the creation of a colored complex between the products of lipid peroxidation and thiobarbituric acid at a temperature of 100 °C in an acidic environment. The maximum absorption of this complex occurs at a wavelength of 532 nm.

b) Determination of the Effect of PA on RS Generation Total RS generation was estimated in culture supernatant. Twenty-five mM 2',7'-dichlorofluorescin diacetate (DCFH-DA) was added to incubation media, and changes in fluorescence were measured at an excitation wavelength of 486 nm and emission wavelength of 530 nm for 30 min.

c) Measurement of NO′ Production NO′ production was estimated by determining the accumulation of nitrite in the conditioned medium by the Griess assay. In short, 100 μl of culture supernatant was added to react with 100 μl of Griess reagent and then incubated at room temperature for 5 min. The optical density at 540 nm of the samples was determined using a Microplate Reader.

d) Measurement of O₂⁻ The O₂⁻ levels were measured following the method described by Ewing and Janero. The O₂⁻-scavenging activity was determined by assessing the decrease in the ratio of the reduction of nitro blue tetrazolium (NBT). Culture supernatant was added to the reaction buffer (50 mM PBS with 125 μM ethylenediaminetetraacetic acid (EDTA), 62 μM NBT and 98 μM NADH) containing 33 μM 5-methylphenazinium methyl sulfate. The absorbance at 540 nm, as an index of NBT reduction, was measured after 5 min.

e) Measurement of ONOO⁻ ONOO⁻-dependent oxidation of dihydrorhodamine 123 (DHR 123) to rhodamine 123 was estimated based on the method described by Kooy et al. Samples were added to the rhodamine buffer (pH 7.4) containing 6.25 mM DHR 123 and 125 mM diethylenetriaminepentaacetic acid (DTDA) and incubated 5 min at 37 °C. The absorbance was measured at 500 nm, which is the absorbance of rhodamine 123.

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

Protein Assay The concentration of protein was assessed by bicinchoninic acid protein assay. All samples were assayed in triplicate.

Statistical Analysis The values are expressed as mean±standard error (n=5) and the biological significance p<0.05 was determined by the Student’s t-test.

RESULTS

Effect of PAs on the Viability of B16 Cells The results
from the cell viability assay using MTT for B16 cells are displayed in Fig. 1. At growth doses of 5, 10, 25 and 50 μg/ml of the PA oligomer revealed 99.8%, 98.9%, 97.3% and 91.5%, respectively, and the PA polymer showed 99.1% at 5 μg/ml, 98.4% at 10 μg/ml, 95.2% at 25 μg/ml, and 90.1% at 50 μg/ml, with 24 h treatment. These results indicated that PAs are relatively non-cytotoxic to cells under the experimental conditions used.

### Suppressive Action of PAs on Murine Tyrosinase Activity

As displayed in Table 1, PAs inhibit murine tyrosinase activity. The low IC\textsubscript{50} value of PA oligomer and polymer (IC\textsubscript{50}=6.3 μg/ml and 22.6 μg/ml, respectively) indicates that the potency was significantly higher than that of kojic acid (IC\textsubscript{50}=73.7 μg/ml), which was used as the positive control.

### Inhibitory Action of PAs on Melanin Synthesis

In the current work, the inhibitory activity of PAs on melanin content is shown in Fig. 2. The melanin content in B16 cells treated with oligomeric PA decreased dose dependently, showing 34.2% at 5 μg/ml, 22.4% at 10 μg/ml, 16.7% at 25 μg/ml and 7.0% at 50 μg/ml, and polymeric PA showed 74.5% at 5 μg/ml, 56.7% at 10 μg/ml, 37.9% at 25 μg/ml and 20.4% at 50 μg/ml when compared to the α-MSH-treated control group.

### Effect of PAs on the Lipid Peroxidation Marker

Figure 3 shows that TBARS levels were significantly decreased in the PA-treated group, indicating that lipid peroxidation was effectively suppressed by the PAs.

### Suppression of RS Generation

The effects of PAs on the total RS generation are shown in Fig. 4. RS generation was significantly reduced by the PA oligomer, by 52.9% at 5 μg/ml, 40.2% at 10 μg/ml, 31.9% at 25 μg/ml and 7.1% at 50 μg/ml, and the PA polymer-treated cells showed suppressed RS of 80.4% at 5 μg/ml, 71.6% at 10 μg/ml, 62.3%

### Table 1. Effects of Oligomer and Polymer PAs, and Kojic Acid on Murine Tyrosinase Activity

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC\textsubscript{50} (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA oligomer</td>
<td>6.3</td>
</tr>
<tr>
<td>PA polymer</td>
<td>22.6</td>
</tr>
<tr>
<td>Kojic acid</td>
<td>73.7</td>
</tr>
</tbody>
</table>
The present work assessed the effects of PAs on the oxidative stress markers, GSH and GSSG and their ratio. Table 3 shows that intracellular GSH levels in B16 cells were augmented by PAs, while GSSG levels were reduced. Consequently, the GSH/GSSG ratio was enhanced by PA treatment, indicating an up-regulated intracellular reducing power that is expected to play a critical role in the regulation of melanin synthesis by maintaining redox balance.

**DISCUSSION**

Our long interest in PAs led to the present investigation of PAs on melanogenesis. To the best of our knowledge, our present work provides the first evidence that PAs isolated from persimmon peel have potent modulatory effect on melanogenesis.

First, we wanted to ascertain the potential cytotoxicity of the PAs on the B16 cells used in this study. The cytotoxic effects of the PAs were determined by measuring cell viability, where found no significant cytotoxic effect at any of the concentrations tested, as shown in Fig. 1.

Table 1 shows the inhibitory action on murine tyrosinase by PA oligomer and polymer (IC50 = 6.3 μg/ml and 22.6 μg/ml, respectively). We compared the inhibitory action of PA on murine tyrosinase with the well-known tyrosinase inhibitor, kojic acid (IC50 = 73.7 μg/ml) which showed that PAs were significantly effective inhibitors. To further explore the action of PAs on melanogenesis, the melanin content was measured in B16 cells. Results clearly showed that PAs suppressed the melanin of B16 cells (Fig. 2). Thus, our current data on tyrosinase agree with our previous reports10,11 and those of others12 that the suppression of melanogenesis is characterized by the inhibition of tyrosinase. The structural requirement responsible for this inhibitory action was shown in our previous studies.10,11 Our data support the notion that the structural importance of hydroxyl moieties localized in the phenolic ring as shown in the tyrosinase inhibitory effects of pycnogenol with multiple hydroxyl groups.12

To find the underlying mechanism for the anti-melanogenic effect of PAs, their actions against oxidative stress were determined. The process of melanogenesis causes the generation of RS, and the occurrence of oxidative stress has been proposed as a pathogenetic mechanism for hyperpigmentation.33 It is likely that the increased oxidative stress from melanogenesis process that may further enhance the tyrosinase activity, thus exacerbating the situation.

One of our interesting findings (Fig. 3) is that controlling lipid peroxidation is a significant factor in melanogenesis. This notion is supported by the previous report of Hachinohe and Matsumoto34 who showed that lipid peroxidation is closely related with melanogenesis. Also, as suggested in our recent study,12 total RS, ‘O2•−, and ONOO− may well be major underlying factors of melanogenic activity. Data in Table 2 indicate that ‘O2•−, NO•−, and ONOO− generation were significantly blunted by PAs, indicating that the suppression of ‘O2•−, NO•−, and ONOO− is a key requirement for the anti-melanogenic effect of the PAs studied.

Failure to control the generation of various RS results in redox imbalance, leading to oxidative stress.35 As Table 3 displays, the enhanced GSH/GSSG ratio by the PAs may likely be responsible for the melanogenic inhibition in B16 cells. GSH is an important regulator of melanin synthesis, and our current data on GSH levels agree with previous reports.9,36

One other interesting finding revealed in our present work needing to be noted is the relative efficacy of two forms of PAs, oligomer and polymer. In our hands, the oligomer PA was the more potent anti-melanogenic (Table 1), as the IC50 value indicated that polymerized PA molecules were far less effective. This finding is in agreement with the interpretation of data on the difference between oligomer and polymer PAs for their relative anti-oxidative efficacy.37,38

Taken together, our present work provides strong evidence to support that the PAs studied inhibited melanogenesis, and that this effect may be related to the modulation of various RS, like ‘O2•−, NO•−, and ONOO−, as well as maintaining redox status in B16 cells. In addition, oligomeric PA showed the stronger anti-melanogenic and anti-oxidant activity. In conclusion, PAs can be useful natural compounds possibly for skin health through their ability to modulate melanogene-
sis and oxidative stress; however, further study is needed to elucidate the exact molecular mechanism by which the benefi
cial effects of PAs work on skin.

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