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ORIGINAL PAPER

Effects of the isoflavone puerarin and its glycosides on melanogenesis in B16 melanocytes

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Abstract We investigated the effects of puerarin (8-C-glucosyl-7,4′-dihydroxy isoflavone), an isoflavone found in Kudzu roots (*Pueraria lobata*), and its glycosides (enzymatically synthesised, water-soluble derivatives of puerarin) on melanogenesis in vitro. Puerarin and its glycosides reduced mushroom tyrosinase activity by 88 and 67% at 4.8 mM, respectively, in a concentration-dependent manner. The puerarin glycosides were less effective than puerarin at the same concentration but showed a comparable inhibitory effect at a concentration at which puerarin is insoluble in water. In cultured B16 melanocytes, the melanin content was reduced significantly; moreover, tyrosinase activity was inhibited significantly by both puerarin and its glycosides when added at a concentration of 480 μM. DNA microarray and RT-PCR analyses showed significant downregulation of the expression of *microphthalmia-associated transcription factor* (*MITF*) and its target genes. The protein expression of MITF and tyrosinase was also downregulated significantly by 40 and 50%, respectively. Our findings suggest that puerarin and its glycosides cause hypopigmentation via dual mechanisms: by inhibiting tyrosinase activity directly and by altering the expression of melanogenesis-related genes, such as *MITF* and tyrosinase. Therefore, puerarin and its glycosides may have potential for the development of functional cosmetics causing hypopigmentation.

Keywords Puerarin · Melanogenesis · Hypopigmentation · Tyrosinase · Melanin · B16 murine melanocytes · Microarray analysis

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Introduction

Pigmentation is highly heritable and is regulated by genetic, environmental, and endocrine factors that modulate the amount, types, and distribution of melanin in the skin, hair, and eyes [1]. Melanocytes in the epidermis interact with keratinocytes and produce melanin pigments in response to both external factors such as ultraviolet (UV) rays, tanning, and photo-ageing chemicals, and internal factors such as autocrine and paracrine cytokines, inflammation, and proinflammatory hyperpigmentation [1].

Hyperpigmentation of the skin causes freckles and liver spots, both of which are considered serious cosmetic problems [2]. The overproduction of melanin is seen in

several medical conditions, including Addison's disease; therefore, adequate control of melanin synthesis is important in normal skin physiology. Melanin synthesis is regulated by a complex network of gene expression and signalling pathways. The melanogenic enzymes tyrosinase, tyrosinase-related protein 1 (TRP-1), and tyrosinase-related protein 2 (TRP-2) are thought to be the major enzymes in melanin biosynthesis [3]. Tyrosinase, the rate-limiting enzyme in melanin synthesis, is a bifunctional enzyme that catalyses the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and mediates the oxidation of DOPA to dopaquinone [4]. TRP-2, which functions as dopachrome tautomerase, catalyses the rearrangement of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA) [5], while TRP-1 oxidises DHICA to a carboxylated indole-quinone, which is eventually converted into eumelanins [6]. Consequently, the direct inhibition of the activities of these enzymes or the downregulation of the genes encoding these enzymes, together with the inhibition of general melanogenic pathways, could be useful in development of products causing skin hypopigmentation. Essential to the regulation of these genes is microphthalmia-associated transcription factor (MITF), a basic helix-loop-helix-zip transcription factor that regulates both melanocyte proliferation and melanogenesis. MITF is a major regulator of tyrosinase, TRPs, and several melanosome structural proteins, including pMel17. Thus, any compound with the ability to reduce *MITF* expression may regulate the function of the transcription factor's target genes in melanin biosynthesis.

Several plant polyphenols show tyrosinase inhibitory effects via the chelation of copper ions at the active site. Tyrosinase inhibition by major soya bean isoflavones such as genistein and its derivatives has similar effects to those of other flavonoids, including garlic acids and cardol derivatives. Kudzu (*Pueraria lobata*), a tuberous plant native to eastern Asia, contains large amounts of isoflavonoids, especially puerarin (8-C-glucosyl-7,4′-dihydroxy isoflavone; Fig. 1) [7]. Kudzu has been used in traditional Oriental medicine for thousands of years and contains various bioactive compounds. The active isoflavone puerarin is found in Kudzu in large quantities (approximately 1% of the plant's dry weight). Puerarin has multiple biological functions, including antioxidative properties [8], hypocholesterolaemic effects [9], and preventive effects on alcoholism [10, 11]. Kudzu is currently used as a phytochemical ingredient in cosmetics and dietary supplements [12]. Puerarin may have melanogenesis-reducing activity like its isoflavone counterparts, although its effects on melanogenesis and hypopigmentation have not been investigated.

Like other isoflavones, puerarin has low water solubility, which limits its bioavailability and industrial applications. Therefore, we previously developed an enzymatic method for synthesising water-soluble puerarin glycosides using a novel transglycosylation enzyme. The water solubility of the puerarin glycosides was improved >300-fold [13], while their antioxidative and hypocholesterolaemic activities remained similar to those of puerarin both in vitro and in vivo [14]. In this study, we further investigated the potential hypopigmentative effects of puerarin and its glycosides using cultured melanocytes treated with an equimolar mixture of maltosyl-α-(1,6)-puerarin and glucosyl-α-(1,6)-puerarin (Fig. 1).

Materials and methods

Reagents

Mushroom tyrosinase, protease inhibitor cocktail, and bovine serum albumin were purchased from Sigma (St. Louis, MO, USA). B16 murine melanocytes were purchased from the Korean Cell Line Bank (Seoul, Korea), while protein quantification kits were obtained from Bio-Rad (Hercules, CA, USA). The ECL™ Western blotting analysis system, Cy3-dUTP, and Cy5-dUTP were obtained from Amersham Biosciences (Piscataway, NJ, USA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). Protran nitrocellulose transfer membrane was purchased from Whatman (Dassel, Germany), while RNeasy Mini Kits were purchased from Qiagen (Hilden, Germany). Oligonucleotide microarrays were purchased from the Stanford Functional Genomics Facility (Stanford, CA, USA). Puerarin was purchased from Shanghai DND Pharm-Tech (Shanghai, China). The purity of the puerarin was >99%, as assessed using high-performance liquid chromatography (HPLC).

Preparation and purification of puerarin glycosides

Puerarin glycosides were prepared and assayed as described by Li et al. [13]. Briefly, maltogenic amylase from *Bacillus stearothermophilus* (BSMA) was used for the transglycosylation reaction using maltotriose as the donor molecule (Fig. 1). Puerarin (1% w/v) and maltotriose (5% w/v) were used as acceptor and donor molecules, respectively. The acceptor-to-donor ratio was optimised empirically. The molecules were dissolved in the optimum buffer then pre-incubated at the optimum temperature for 10 min. Next, BSMA (50 U/mL) was added. The reaction was stopped by boiling for 10 min after a 45-min incubation and filtered through a 0.45-μm membrane (PVDF, Millipore, Billerica, MA, USA) prior to purification. A Sep-Pak Plus C18 cartridge, previously activated with ethyl acetate, methanol (MeOH), and water, was used to absorb the

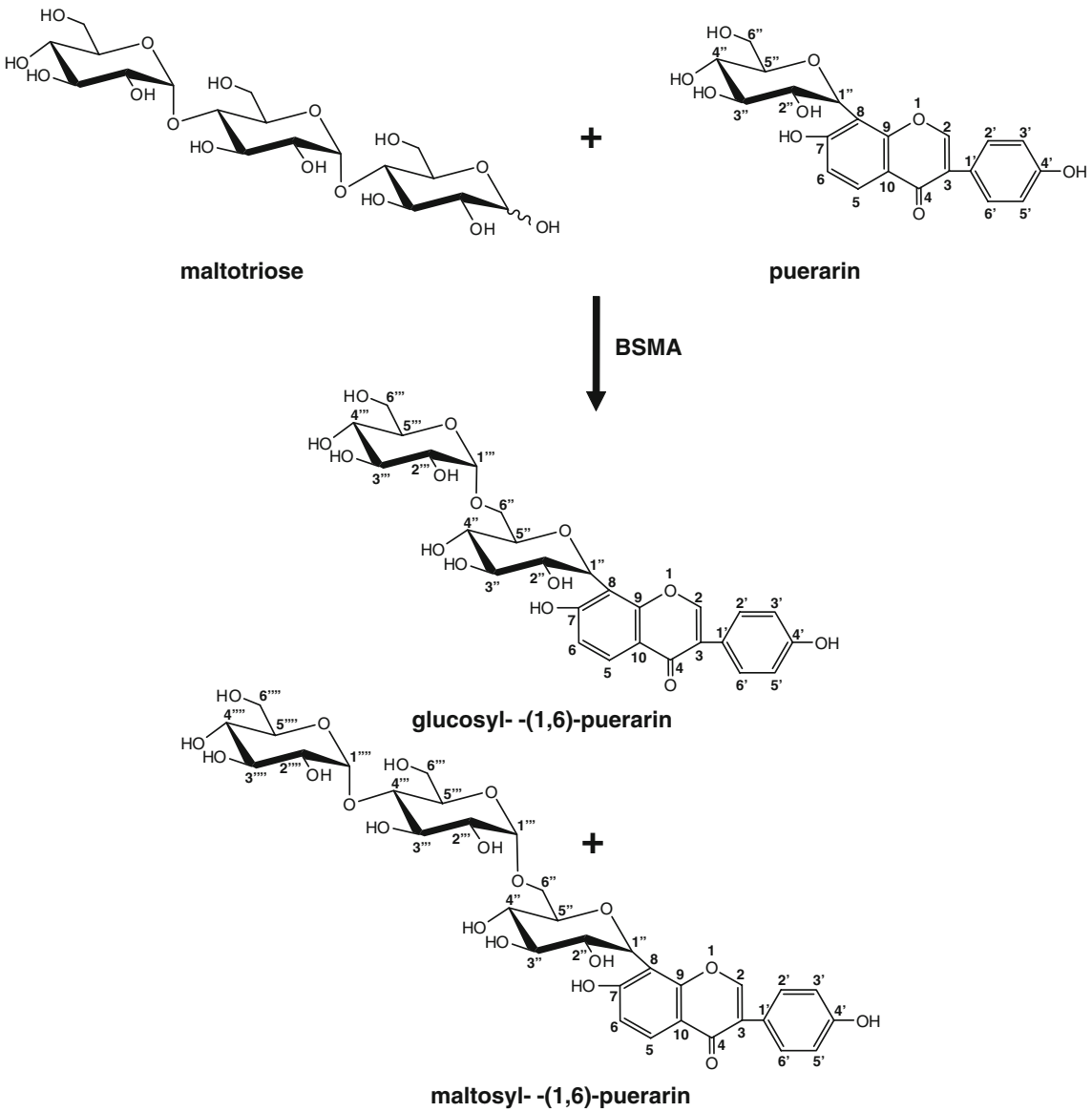

Fig. 1 Production of puerarin glycosides using *Bacillus stearothermophilus* maltogenic amylase (BSMA) from puerarin (8-C-glucosyl-7,4′-dihydroxy isoflavone) and maltotriose. The puerarin glycosides were determined to be maltosyl-α-(1,6)-puerarin (G2-puerarin) and glucosyl-α-(1,6) -puerarin (G1-puerarin) by HPLC

puerarin glycosides in the transglycosylation solution and to remove the remaining maltooligosaccharide mixture and salt. The solution eluted with MeOH was purified using a polymeric gel-filtration column (W-251) with recycling preparative HPLC to obtain the final transglycosylation products. The mobile phase (50% [v/v] MeOH) was applied at 2 mL/min.

After separation of the transglycosylated products, G2-puerarin [maltosyl-α-(1,6)-puerarin] and G1-puerarin [glucosyl-α-(1,6)-puerarin] were fractionated. Each puerarin glycoside peak produced a single spot by thin-layer chromatography and a single peak on the HPLC chromatogram (data not shown). The structures of the compounds were confirmed using ESI^+ TOF–MS–MS and C^{13} NMR spectroscopy and have been published [13], and α-(1,6)-glycosidic linkage between sugar moiety and puerarin was doubly confirmed with incubation with TreX, a debranching enzyme that specifically cleaves α-(1,6)-glycosidic linkage but not β-(1,6)-glycosidic linkage (data not shown). Since the protocol produced limited amounts of each glycoside, we mixed equal numbers of moles of G1- and G2-puerarins based on their respective molecular weights. As the molecular weight of G1-puerarin is 578 g/mol while that of G2-puerarin is 740 g/mol, the average molecular weight of the glycosides was found to be $(578 + 740)/2 = 659$ g/mol. The resulting mixture of

puerarin glycosides was used to screen for potential hypopigmentative effects.

Mushroom tyrosinase assay

The DOPA oxidase activity of mushroom tyrosinase was measured spectrophotometrically as described previously with minor modifications [15]. Fifty microlitres of 0.03% tyrosine in distilled water and 75 μL of 0.1 M phosphate buffer (pH 6.8) with or without the test compounds were added to a 96-well microplate, and 25 μL of mushroom tyrosinase (400 μL/mL) in 0.1 M phosphate buffer was added. The reaction was incubated at 37 °C for 20 min. The yield of dopachrome was determined spectrophotometrically at 492 nm (Microplate Manager, Bio-Rad). The inhibitory effects of the test samples on the enzyme were calculated as the per cent inhibition (i.e. [1 − (sample OD_{492}/control OD_{492})] × 100).

Cell culture

B16 murine melanocytes were cultured in Dulbecco's modified Eagle's medium containing 10% foetal bovine serum, 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37 °C in a humidified atmosphere consisting of 95% air and 5% CO_2.

MTT assay

The general viability of the cultured cells was determined based on the amount of 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium bromide (MTT) reduced to formazan. After treatment, culture medium containing MTT (0.5 mg/mL) was added to each well, and the cells were incubated at 37 °C for 3 h before being mixed with dimethyl sulphoxide to dissolve the formazan crystals. Next, the absorbance was measured at 570 nm using a spectrophotometer (Microplate Manager, Bio-Rad). The values were normalised using the protein concentrations.

B16 melanocyte tyrosinase assay

Tyrosinase activity was assayed as DOPA oxidase activity using the method described previously [15]. The cells were washed twice with ice-cold phosphate-buffered saline (PBS) and lysed with 20 mM Tris-0.1% Triton X-100 (pH 7.5). Tyrosinase activity was then analysed spectrophotometrically. The concentration of dopachrome in the reaction mixture was measured at 475 nm. The reaction mixture, containing 140 μL of freshly prepared substrate solution [0.1% L-DOPA in 0.1 M sodium phosphate (pH 6.0)] and 70 μL of enzyme solution, was incubated at 37 °C. The change in absorbance was measured for the first 2 h of the reaction (i.e. while the increase in absorbance was linear). Corrections were made for the auto-oxidation of L-DOPA in the controls. Enzymatic activity was expressed as the percentage of that in the control cells. The values were normalised against the protein content of the samples. The protein concentration was determined by the Bradford method using a commercial kit (Bio-Rad).

Melanin content

To determine the melanin content of the cells, B16 cells were firstly washed with PBS then lysed with 20 mM Tris-0.1% Triton X-100 (pH 7.5). After centrifugation at 12,000 × *g* for 5 min at 4 °C, the pellet was dissolved in 1 N NaOH for 1 h at 60 °C. The absorbance was then measured at 400 nm, and the melanin content was determined using synthetic melanin as the standard. The amount of cellular melanin was corrected against the protein content of the samples, which was determined using the Bradford method.

Microarray analysis

B16 cells were treated with or without puerarin at a concentration of 480 μM for 12 h then subjected to microarray analysis using total RNA isolated from the cells with TRIzol reagent (Invitrogen) and purified with an RNeasy Mini Kit (Qiagen). To remove any contaminating DNA, on-column digestion with RNase-Free DNase (Qiagen) was performed. Eight micrograms of purified total RNA was used for cDNA synthesis with T7-(deoxythymidine) 24-mer and Superscript II (Invitrogen); the cDNA was subsequently labelled with Cy3-dUTP or Cy5-dUTP (Amersham Biosciences). The labelled cDNA samples were hybridised with 38.8-K oligonucleotide arrays containing 30,105 constitutive exonic probes. The arrays were then scanned with a GenePix 4000B (Axon Instruments, Palo Alto, CA, USA), and the data were visualised using GenePix 4.0 software (Axon Instruments). The hybridisation procedure was repeated with six array chips. R statistical freeware (available at http://www.r-project.org/) combined with the limma package was used for data normalisation, which included background correction within and between array normalisations (Supplementary Fig. 1a). To analyse the relationship between dye-bias and intensity, M versus A plots, and fluorescence intensity density plots were drawn between normalisation steps (Supplementary Fig. 1b). Significant genes were detected using a modified *t*-test, significance analysis of microarrays (SAM) (Supplementary Fig. 1c). Upregulated and downregulated genes were extracted from the normalised data. Candidate melanogenesis-related genes were selected, and fold-changes in expression were calculated. To avoid the low-variance problem inherent in *t*-tests, SAM uses a

statistical method similar to the *t*-test and estimates the false discovery rate (FDR) from the permutation of repeated measurements [16]. In our analysis, a delta value of 1.3 (FDR = 0.0049) was used (Supplementary Fig. 1c). Differentially expressed genes were also analysed using AmiGo gene ontology lists [17]. Each gene was confirmed to have significant hepatic expression using the Gene Expression Database of the Mouse Genome Informatics online resource [18] and the UniGene database [19].

RT-PCR

Total cellular RNA was isolated using TRIzol reagent. To synthesise cDNA, 2 μg of mRNA from melanocytes was reverse-transcribed with oligo dT and Superscript II as recommended by the manufacturer (Invitrogen). The reaction was performed in a 20-μL volume for 90 min at 42 °C and subsequently at 70 °C for 15 min. The resulting cDNA templates were amplified by PCR in a mix containing cDNA, 10× PCR buffer, 2.5 mM dNTPs, 10 mM forward and reverse primers, DNA polymerase, and sterile water. The cDNA was also used for RT-PCR using primers specific for *tyrosinase*, *TRP-1*, and *TRP-2* (Bioneer, Daejeon, Korea). The sequences of the primers were as follows: *tyrosinase* upstream 5′-GGC CAG CTT TCA GGC AGA GGT-3′ and downstream 5′-TGG TGC TTC ATG GGC AAA ATC-3′; *TRP-1* upstream 5′-GCT GCA GGA GCC TTC TTT CTC-3′ and downstream 5′-AAG ACG CTG CAC TGC TGG TC T-3′; and *TRP-2* upstream 5′-GGA TGA CCG TGA GCA ATG GCC-3′ and downstream 5′-CGG TTG TGA CCA ATG GGT GCC-3′. Primers specific for *β-actin* (upstream 5′-TGC TGT CCC TGT ATG CCTCT-3′ and downstream 5′-AGG TCT TTA CGG ATG TCA ACG-3′) were added as a control for the same reverse-transcription product. The PCR products (10 μL) were electrophoresed on 1.2% agarose gels at 100 V and stained with ethidium bromide before visualisation using ultraviolet light. The RT-PCR products were quantified by measuring the intensity of each band using GelPro analysis software.

Western blot assays

Cells were lysed in RIPA buffer (10 mM Tris–HCl, pH 7.5, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, and 1 mM EDTA) containing protease inhibitor cocktail (Sigma) at 4 °C. The lysate was clarified by centrifugation at 12,000×*g* for 5 min at 4 °C. The protein concentration was then determined using a Bio-Rad protein kit with bovine serum albumin (Sigma) as a standard. Equal amounts of protein were boiled in sample buffer (5% *β*-mercaptoethanol) for 5 min. The samples were then separated by 7.5% sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and blotted onto a nitrocellulose membrane (0.45 μM). Non-specific binding was blocked by incubation in PBS, pH 7.4, 0.1% Tween 20, and 5% skim milk for 1 h at room temperature. Primary antibodies raised against tyrosinase, TRP-1, and TRP-2 were used at 1:500 dilutions, prepared in fresh blocking buffer. All antibodies were incubated overnight at 4 °C. After several washes with PBS-0.1% Tween 20, the membrane was incubated with anti-goat IgG, H&L chain-specific peroxidase conjugated secondary antibodies (1:5,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoreactive bands were detected using the ECL™ Western blotting analysis system (Amersham Biosciences) and exposed to high-performance chemiluminescence film for 30 s. The immunoblots were scanned by a 690 Bio-Rad Densitometer using the Multi-Analyst program (Bio-Rad). The density of each band was quantified using GelPro analysis software.

Statistical analysis

Each experiment was repeated at least three times. Student's *t*-test was used for comparisons between groups. All results were presented as the mean ± SEM unless specified. Analysis of variance (ANOVA) was used for multiple comparisons. Values were considered to be significant at $p < 0.05$.

Results

Inhibition of the DOPA oxidase activity of mushroom tyrosinase by puerarin and its glycosides

The effect of puerarin on the DOPA oxidase activity of mushroom tyrosinase was examined. Puerarin inhibited the DOPA oxidase activity of mushroom tyrosinase directly in a dose-dependent manner (Fig. 2). At concentrations between 100 and 4,800 μM, puerarin and its glycosides reduced the level of DOPA oxidase activity by 5 to 88%, respectively. Tyrosinase inhibition by puerarin isoflavones was comparable to that by arbutin (arbutoside hydroquinone *β*-D-glucopyranoside) at the same molar concentrations, while it was weaker than that by phenylthiourea (PTU). PTU strongly inhibits tyrosinase activity by chelating copper ions, which function as a cofactor for the enzyme [20]; thus, it was included as a positive control in our subsequent experiments.

Effects of puerarin and its glycosides on B16 melanocyte cell viability, melanin content, and tyrosinase activity

To study the effect of puerarin and its glycosides on melanogenesis in vitro, murine B16 melanoma cells were

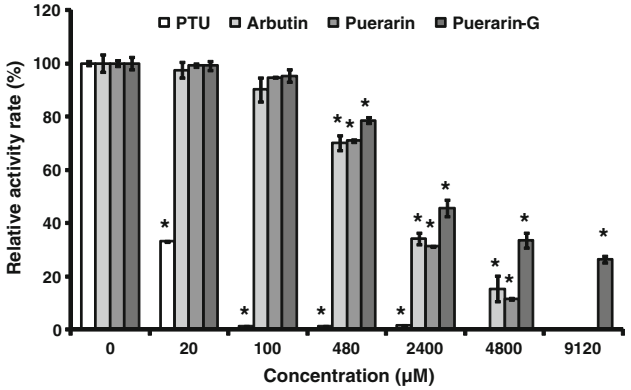

Fig. 2 Effects of puerarin and its glycosides on mushroom tyrosinase activity. The data are expressed as the mean ± SEM. *PTU* phenylthiourea, *puerarin-G* puerarin glycosides, * $p < 0.05$ compared with zero μmol

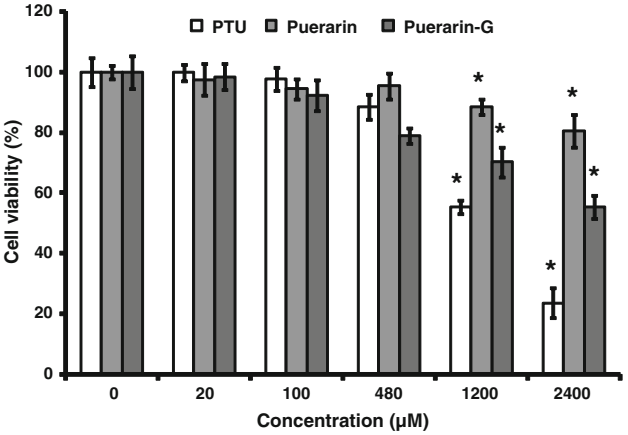

Fig. 3 Concentration effects of puerarin and its glycosides on the viability of B16 murine melanocytes as determined using the MTT assay. The values were normalised based on the protein concentrations. The data are given as the mean ± SEM. *PTU* phenylthiourea, *puerarin-G* puerarin glycosides, * $p < 0.05$

incubated with various concentrations of puerarin and its glycosides. The potentially toxic effect of puerarin was assessed using the MTT assay. Puerarin and PTU marginally affected the viability of B16 cells at concentrations up to 480 μM (Fig. 3); however, PTU, but not puerarin, was toxic when provided in amounts exceeding 480 μM. The inhibitory effects of puerarin on melanocyte tyrosinase and the melanin content were measured in B16 melanoma cells. The cellular melanin content was decreased by 22.0% ($p < 0.05$) and 25.0% ($p < 0.05$) by puerarin and its glycosides at 480 μM (Fig. 4a). Puerarin and its glycosides caused a slight but significant reduction in tyrosinase activity at 96 and 480 μM (29.6% [$p = 0.01$] and 9.6% [$p = 0.05$] at 480 μM of puerarin and its glycosides, respectively; Fig. 4b). These data confirm that puerarin and its glycosides inhibit tyrosinase activity directly and are in line with our mushroom tyrosinase results.

Genome-wide effects of the isoflavone puerarin on B16 murine melanocytes

Six DNA microarrays were used to investigate the genome-wide effects of the isoflavone puerarin on B16 cells. Treatment of B16 cells with puerarin at a concentration of 480 μM for 12 h caused minor changes in the expression profile of multiple genes. A total of 897 genes were significantly altered: 559 were upregulated and 338 were downregulated (FDR = 0.0049). This supports the notion that phytochemicals induce small changes in the expression of multiple genes that could have significant net effects on cellular physiology. Based on their gene ontology (GO) annotations, the 559 upregulated genes were found to belong to multiple functional biological pathways, including those involved in the positive regulation of keratinisation, epidermal development, signal transduction, and cell adhesion. The downregulated genes were involved in

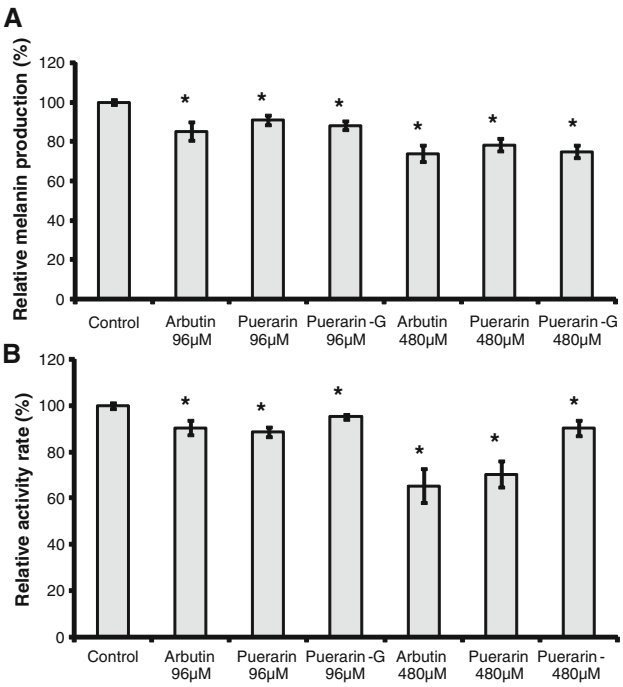

Fig. 4 Effects of puerarin and its glycosides on the melanin content and cellular tyrosinase activity. The values were normalised based on the protein concentrations. **a** Melanin content. **b** Tyrosinase activity. The data are expressed as percentages compared to the controls and are shown as the mean ± SEM. *P* puerarin, *puerarin-G* puerarin glycosides, * $p < 0.05$. Phenylthiourea (PTU) was used as a reference

pathways regulating tissue development, the protein kinase cascade, and cellular differentiation (Supplementary Fig. 2). Melanogenesis-related gene expression in melanocytes was assessed based on our microarray results. The expression of endothelin B receptor (ET_B-14%) and its downstream target protein kinase C (*PKC*, +22%) was

Table 1 Transcript expression of melanogenesis genes in melanocytes monitored using microarray hybridisation

Gene name (gene symbol)	Functions	Average fold change
Melanocortin-1 receptor (*Mc1r*)	Receptor for α-MSH, ACTH	0.74
Endothelin B receptor (*Etbr*)	Receptor of ET-1	0.86
cAMP-dependent protein kinase (*PKA*)	Activating MITF through phosphorylation of CREB	0.59
Protein kinase C (*PKC*)	Phosphorylation activates tyrosinase expression	1.22
Microphthalmia-associated transcription factor (*MITF*)	Transcription factor induces tyrosinase, TRP-1, and TRP-2	0.70
Tyrosinase-related protein 1 (*TRP-1*)	Melanin biosynthesis	0.53
Tyrosinase-related protein 2 (*TRP-2*)	Melanin biosynthesis	0.54

* The tyrosinase (TYR) gene was not spotted on the tested arrays

α-MSH α-melanocyte-stimulating hormone, *ACTH* adrenocorticotropic hormone, *ET-1* endothelin-1

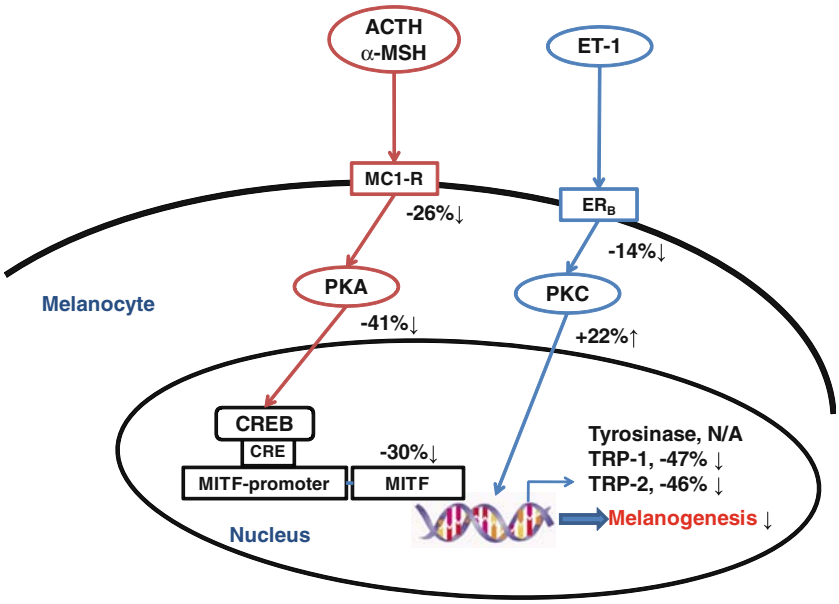

Fig. 5 Effects of puerarin on melanocortin 1 receptor (MC1-R)- and endothelin B receptor (ET_B)-mediated signalling. *PKA* protein kinase A, *PKC* protein kinase C, *CREB* cAMP response element binding protein, *MITF* microphthalmia-associated transcription factor, *TRP* tyrosinase-related protein. The percent values indicate the average gene expression changes following puerarin treatment by microarray analysis

regulated in opposite directions by puerarin, while the expression of *melanocortin-1 receptor* (*MC1-R*) and *protein kinase A* (*PKA*) was downregulated significantly by 26 and 41%, respectively (Table 1; Fig. 5). This may be the major mechanism underlying the downregulation of MITF, TRP-1, and TRP-2 expression resulting in hypopigmentation in B16 cells. MITF, TRP-1, and TRP-2 were significantly downregulated by 30, 47, and 46%, respectively (Table 1; Fig. 5). The gene encoding tyrosinase was not spotted on the array.

Effects of puerarin on melanin-biosynthetic genes

We further investigated the expression of MITF and its target genes, including *tyrosinase*, *TRP-1*, and *TRP-2*, using RT-PCR and immunoblot analysis. We examined the effects of high (480 μM) and low (96 μM) concentrations of puerarin. At 480 μM, puerarin reduced the mRNA expression of *tyrosinase* and *TRP-1* (Fig. 6a), while at 96 and 240 μM it reduced the protein expression of tyrosinase by 46 ($p = 0.20$) and 50% ($p < 0.05$), respectively (Fig. 6b). We also measured the changes in MITF protein expression following puerarin treatment. MITF is a transcription factor that activates the expression of genes related to melanogenesis. We found that MITF expression was reduced by 40% in B16 cells after incubation with puerarin (240 μM).

Discussion

We investigated the effects of puerarin and its glycosides on melanogenesis. Puerarin and its glycosides significantly suppressed the activity of mushroom tyrosinase at levels similar to arbutin and were not toxic to cultured B16 melanocytes when supplied at concentrations of up to 480 μM. Both puerarin and its glycosides reduced the cellular melanin content and significantly inhibited cellular

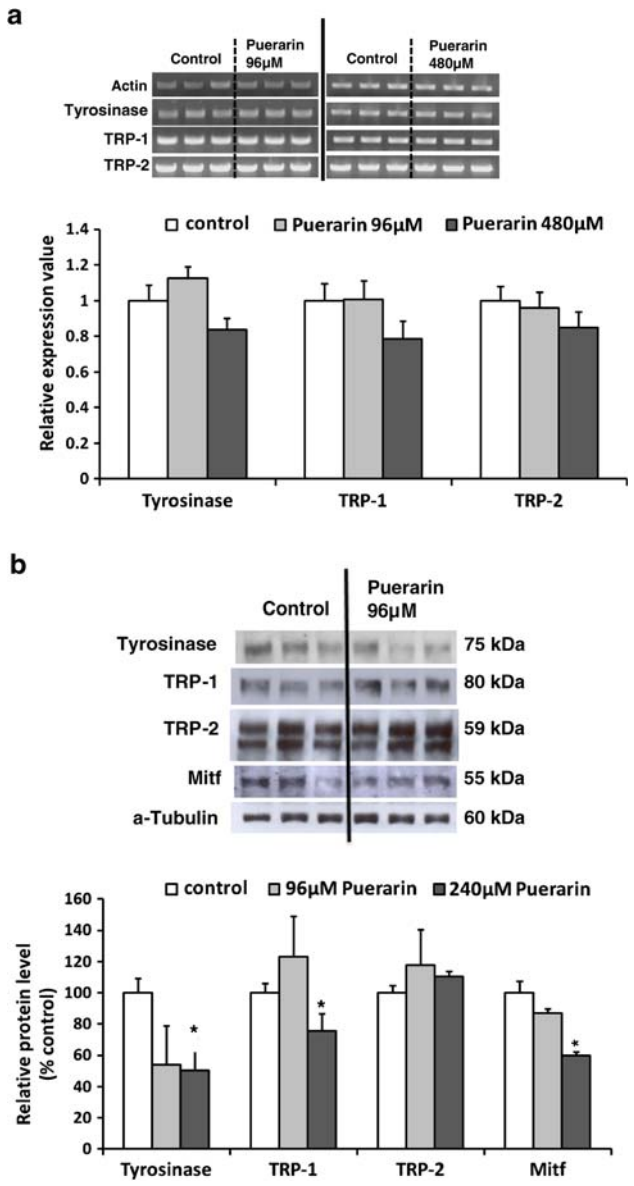

Fig. 6 Expression of melanogenesis-related genes in melanocytes. **a** The expression of *tyrosinase*, *TRP-1*, and *TRP-2* was assessed by RT-PCR. **b** The protein expression of tyrosinase, TRP-1, TRP-2, and MITF was examined by Western blotting

tyrosinase activity in vitro, although puerarin was more effective than its glycosides at the same concentrations. Treatment of B16 cells with puerarin affected both the gene and protein expression of *tyrosinase*, *TRP-1*, and *TRP-2*. These effects were examined using microarray, RT-PCR, and immunoblot analyses. Our data suggest that puerarin protects against pigmentation in melanocytes in two ways: by inhibiting tyrosinase activity directly and by regulating melanogenesis-related gene and protein expression.

Tyrosinase catalyses the first two steps in melanin synthesis: namely the hydroxylation of L-tyrosine to L-DOPA and the oxidation of L-DOPA to dopaquinone [21]. The inhibition of monophenolase or diphenolase activity could be effective in treating hyperpigmentation [22]. The inhibition of tyrosinase activity has been the subject of many studies, and there has been a concerted effort to search for naturally occurring tyrosinase inhibitors from plants. Several isoflavone derivatives have been shown to inhibit tyrosinase activity [23, 24]. Daidzein, glycetein, daidzin, and genistin are isoflavones from soya bean that have either a 7,8,4′-trihydroxyisoflavone or 5,7,8,4′-tetrahydroxyisoflavone structure [25, 26]. These isoflavones inhibit both the monophenolase and diphenolase activities of tyrosinase [25, 26]. In our study, puerarin and its glycosides significantly inhibited tyrosinase activity. Since puerarin has a daidzein-like structure, we postulate that puerarin inhibits tyrosinase activity via a mechanism similar to that of daidzein. It has been suggested that the 8-hydroxy group, but not the 8-hydrogen, on the isoflavone ring is associated with greater inhibition [25]. Consequently, the bulky glucose moiety at position 8 of the puerarin ring may be associated with the potent inhibitory effect of puerarin on tyrosinase activity. Additional studies are needed to elucidate the mechanism of tyrosinase inhibition by puerarin.

To explore the genome-wide effects of puerarin on melanocytes, we performed a microarray-based analysis. A list of genes involved in melanogenesis was analysed separately and compared to our microarray results. Melanogenesis can be stimulated by multiple pathways in response to several environmental and hormonal factors. Our microarray results showed that the transcription of *MC1-R* and *PKA* was reduced significantly in line with the downregulation of *MITF* [27, 28]. In human epidermis, the hormones α-MSH [29] and ACTH [30] act via the melanocyte-specific receptor MC1-R, which activates adenylate cyclase through a G-protein, which then produces cAMP from adenosine triphosphate. Cyclic AMP exerts its effect in part through PKA, which phosphorylates and activates cAMP response element binding protein (CREB), which binds to the cAMP response element (CRE) present in the M promoter of *MITF* (Fig. 5) [31]. Our microarray results showed that PKA expression was also downregulated by 41%. PKA has various functions and is largely regulated by post-translational phosphorylation; however, the reduction in PKA may contribute to the expression of *MITF* and melanogenesis-related genes, such as *tyrosinase* and *TRP*s. Based on our microarray results, the expression of *MC1-R*, *PKA*, and *MITF* was downregulated significantly by 26, 41, and 30%, respectively. These results suggest that the downregulation of *MITF* caused the reduction in tyrosinase and TRP-1 expression and *MITF* downregulation is correlated with the expression of MC1-R and PKA. The expression of *TRP-1* and *-2* according to our microarray data was similar to that seen in our RT-PCR results, and *MITF* expression was also significantly reduced by about

30%. The results of this pathway analysis suggest that puerarin enhances the cell cycle and hair and skin development while inhibits cellular proliferation, dermatological diseases, and the immune response (Supplementary Fig. 2). Overall, the effects of puerarin on melanocytes appear to be beneficial for skin health.

In conclusion, we showed that puerarin can reduce melanin formation in melanocytes mainly via the inhibition of tyrosinase, a key enzyme in melanogenesis. Puerarin glycosides, which are soluble derivatives of puerarin, were as effective as puerarin in tyrosinase inhibition. In addition, puerarin reduced tyrosinase and TRP-1 expression by downregulating the transcription factor MITF. MC1-R and PKA expression was positively correlated with MITF expression. These results suggest that puerarin and its glycosides may be useful in anti-melanogenic cosmetics. Additional studies are needed to confirm the hypopigmentation caused by and potential toxicity of puerarin in vivo.

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