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Anemonin is a natural bioactive compound that can regulate tyrosinase-related proteins and mRNA in human melanocytes

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KEYWORDS
Anemonin;
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Summary

Background: Melanin is the pigment responsible for skin color. Melanin synthesis occurs with the participation of the tyrosinase (TYR) family of proteins including TYR, tyrosinase-related protein 1 (TRP1), and tyrosinase-related protein 2 (TRP2/DCT).
Objective: The effect of a newly isolated natural compound that inhibits hyperpigmentation on the regulation of the TYR family of proteins was examined.
Methods: The natural compound, anemonin, was isolated from *Clematis crassifolia* Benth and was used to inhibit cellular TYR activity; it was found to have a low cytotoxicity (cell viability $>$ 80%) in human melanocytes.
Results: In human melanocytes, anemonin showed both time- and dose-dependent inhibition (IC_{50} 43.5 μM) of TYR. Western blot analysis and immunocytochemical staining revealed that expression of TYR, TRP1, and TRP2 was decreased in anemonin-treated melanocytes. Additionally, reverse transcription and quantitative real-time polymerase chain reaction analyses revealed that expression of mRNAs for *MITF*, *TYR*, *TYRP1*, and *TYRP2* was also suppressed by anemonin.
Conclusion: The natural compound, anemonin, an active compound of *C. crassifolia*, inhibits pigmentation synthesis in human melanocytes. Anemonin inhibits melanin synthesis by inhibiting the transcription of the genes encoding *MITF*, *TYR*, *TRP1*, and *TRP2*. This natural compound may be a candidate for cosmetic use.

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1. Introduction

Melanin is the pigment responsible for the color of human skin. The regulation of pigmentation in mammals is a complex process that is controlled by different factors. Melanogenesis is regulated at the subcellular level; the synthesis and expression of various melanogenic enzymes and inhibitors play critical roles in melanogenesis [1]. Tyrosinase (TYR) is known to be a key enzyme that catalyzes the synthesis of melanin in melanocytes [2]. TYR catalyzes two major reactions: the hydroxylation of tyrosine to 3,4-L-dihydroxyphenylalanine (dopa) and the oxidation of dopa to dopaquinone [3]. Dopaquinone spontaneously converts to dopachrome. Tyrosinase-related protein 2/dopachrome tautomerase (TRP2/DCT) catalyzes the conversion of dopachrome to 5,6-dihydroxyindole-2-carboxylic acid (DHICA). Tyrosinase-related protein 1 (TRP1; DHICA oxidase) catalyzes the oxidation of DHICA to indole-5,6-quinone-2-carboxylic acid. These two closely related structures, TRP2/DCT and TRP1, act to produce unstable quinones that undergo further polymerization, which finally results in the production of melanin [4–6].

Tyrosinase and its related proteins are the products of distinct genes that belong to the *TYR* gene family whose 5′-flanking region possesses consensus sites for transcription factors [7]. The human genes that encode the TRPs are often called *TYRP*s due to the similarity of human TRP2/DCT and TRP1 with TYR. The *TYRP1* gene is currently thought to encode DHICA oxidase activity, whereas the *TYRP2* gene encodes dopachrome tautomerase (TRP2/DCT) [8]. Human TYR is encoded by the *TYR* gene. The three *TYRP* human genes are regulated by an upstream microphthalmia-associated transcription factor (*MITF*) that affects gene expression [9]; they are thought to have the potential for functional polymorphisms, which could explain the natural variation in pigmentation phenotypes as well as the existence of several hypopigmented states.

In the development of new skin care drugs, considerable effort has been expended in the search for natural substances; their use in the development of skin care cosmetics has recently been emphasized [10]. It has thus become of great interest to know whether plant compounds have useful activities that could be exploited in modern cosmetic formulations. Anemonin, the dilactone of cyclobutane-1,2-diol-1,2-diacrylic acid, has been isolated from *Pulsatilla chinensis* [11], *Drymaria diandra* [12], *Knowltonia capensis* [13], *Clematis chinensis* [14], and *Ranunculaceous* plants [15,16]. Anemonin has been shown to have anti-inflammatory [17], antibacterial, antiviral, antitoxic, and cytopathogenic properties [18]. *Clematis crassifolia* Benth (Ranunculaceae) is a plant indigenous to Taiwan [19], a country that has an abundance of plant species. In the present study, anemonin was isolated from *C. crassifolia* and tested for cellular anti-TYR activity, its ability to inhibit melanin production, and its effects on TYR and TRPs expression in human epidermal melanocytes.

2. Materials and methods

2.1. Reagents

Triton X-100, L-3,4-dihydroxyphenylalanine (L-dopa), 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), ethylenediaminetetraacetic acid (EDTA), polyacrylamide, aprotinin, and leupeptin were purchased from Sigma (St. Louis, MO, USA). The other chemicals and reagents used in the study were high-grade commercial products.

2.2. Isolation and purification of anemonin

The fresh leaves of *C. crassifolia* (1 kg) were extracted with 100% MeOH at room temperature. The 100% MeOH extract was then filtered and concentrated under reduced pressure, and a suspension of the extract in 85% MeOH was partitioned with *n*-hexane, ethyl acetate (EtOAc), and water-saturated *n*-butanol (*n*-BuOH), respectively. The EtOAc layer was further chromatographed over Sephadex LH-20 and eluted with MeOH to give 11 fractions. Fraction 7 was further purified by semipreparative high-performance liquid chromatography on an ODS column (4.6 mm × 250 mm; flow rate 2.85 ml/min) with acetonitrile/H_2O (15:85) containing 0.1% trifluoroacetic acid (3:7) to give the active compound (75 mg). The compound's structure was elucidated using one- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy. Spectral data were compared with data from the literature [20].

Anemonin has the following characteristics: ^{1}H NMR (CD_3OD, 500 MHz), δ 8.08 (2H, d, J = 5.3 Hz, H-4, H-4′), 6.18 (2H, d, J = 5.3 Hz, H-3, H-3′), 2.61 (2H, m, H-6a, H-6a′), 2.35 (2H, m, H-6b, H-6b′); ^{13}C NMR (CD_3OD, 125 MHz) δ 24.3 (C6, C-6′), 91.9 (C5, C-5′), 121.1 (C3, C-3′), 156.4 (C4, C-4′), 173.2 (C2, C-2′).

2.3. Cell culture

Human melanocytes (Cat. No. C-102-5C; Cascade Biologics, Inc., Portland, OR, USA) obtained from neonatal foreskin were grown in Medium 254, which is a basal medium containing essential and nones-

sential amino acids, vitamins, other organic compounds, trace minerals, and inorganic salts (Cat. No. M-254-500), supplemented with Human Melanocyte Growth Supplement, which contains 0.5% fetal bovine serum, 3 ng/ml basic fibroblast growth factor (human recombinant), 0.2% bovine pituitary extract, 3 μg/ml heparin, 0.18 μg/ml hydrocortisone, 5 μg/ml insulin, 5 μg transferrin, and 10 ng/ml phorbol 12-myristate 13-acetate (Cat. No. S-002-5).

2.4. Cell viability assay

The cell viability of melanocytes was determined using the MTT method. The cells were plated at 10^5 per well (24-well plates). After 24 h of culture, test samples were added, and the cultures were incubated for an additional 24 h. The optical density was measured at 550 nm on a μQuant microplate reader (Bio-Tek Instruments, Inc.). The viability of the melanocytes was calculated using the following formula: (absorbance of sample tested/absorbance of medium only) × 100%.

2.5. Assay of cellular tyrosinase activity

Cellular TYR activity was measured as described previously, with slight modification [21]. The melanocytes (10^5) were cultured in 24-well plates for 24 h. After being treated with the individual test samples for another 24 h, the cells were washed with potassium phosphate-buffered saline (PBS) and lysed with PBS, pH 6.8, containing 1% Triton X-100. The cells were ruptured by freezing and thawing. Then, the lysates were clarified by centrifugation at 10,000 × *g* for 10 min. The protein content was determined using a BCA Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL, USA). After quantifying protein levels, concentrations were adjusted with lysis buffer until each lysate contained the same amount of protein (40 μg). Each well of the 96-well plate contained the lysate, 2.5 mM L-dopa, and 0.1 M PBS, pH 6.8. After incubation at 37 °C for 1 h, the absorbance was measured at 475 nm using the μQuant microplate reader.

2.6. Measurement of melanin content in melanocytes

Melanin content was measured as described previously, with slight modification [22]. The cells were treated with individual tested preparations for 24 h. Cell pellets were dissolved in 1 N NaOH at 37 °C overnight and centrifuged for 10 min at 10,000 × *g*. The optical densities of the supernatants were measured at 450 nm using the μQuant microplate reader.

2.7. Western blot analysis

Western blot analysis was performed as described previously [23]. The cells (10^6) were collected and lysed with iced PBS containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mg/ml aprotinin, and 10 mg/ml leupeptin. The cell lysates were subjected to centrifugation at 12,000 × *g* for 10 min, and the supernatant protein was quantified with a BCA Protein Assay Kit (Pierce Biotechnology, Inc.). Samples (approximately 10 μg of protein) were added to equal volumes of sodium dodecyl sulfate (SDS) sample buffer and boiled for 5 min prior to separation by 10% SDS–polyacrylamide gel electrophoresis (PAGE). Following electrotransfer to polyvinylidene fluoride (PVDF) membranes (Immobilon-P; Millipore Corp., Bedford, MA, USA), the membranes were incubated overnight with blocking solution containing 5% nonfat dry milk, 0.1% Tween 20, and 0.1% NaN_3. Individual anti-TYR (C-19), anti-TRP1 (G-17), and anti-TRP2 (D-18) antibodies (Santa Cruz Biotechnology, Inc.) served as primary antibodies in 1:1000 dilution and were incubated with the PVDF membrane at room temperature for 3 h. After extensive washes, the blots were incubated with alkaline phosphatase-conjugated anti-goat IgG (Santa Cruz Biotechnology) in 1:5000 dilution for 2 h at room temperature. The alkaline phosphatase activity was detected with nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indolyl phosphate (BCIP) substrate. β-Actin was used as the internal control. Each band's related intensities were calculated for each intensity value (intensity × area) using Quantity One 1-D Analysis Software (Bio-Rad, UK); the values were normalized with the control's intensity value.

2.8. RNA isolation and reverse transcription

Total RNA was isolated using the High Pure RNA Isolation Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions. The quality of the total RNA was evaluated using the A260/A280 ratio. To prepare a cDNA pool from each RNA sample, total RNA (1 μg) was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Molecular Biochemicals). Each cDNA pool was stored at −20 °C until quantitative real-time (q-RT) polymerase chain reaction (PCR) or reverse transcription (RT)-PCR analysis was done.

2.9. PCR primers

Specific oligonucleotide primer pairs used for q-RT PCR were selected from the Roche Universal Probe-

Table 1 The sequences of the primers of the *MITF*, *TYR*, *TYRP*s, and *GAPDH*

	Forward primer	Reverse primer
MITF	CCGTCTCTCACTGGATTGGTG	CGTGAATGTGTGTTCATGCCTGG
TYR	CATTCTTCTCCTCTTGGCAGA	CCGCTATCCCAGTAAGTGGA
TYRP1	GCTTTTCTCACATGGCACAG	GGCTCTTGCAACATTTCCTG
TYRP2	CGACTCTGATTAGTCGGAACTCA	GGTGGTTGTAGTCATCCAAGC
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC

Library. ProbeFinder software (www.universalprobelibrary.com) was used to design the optimal assay composed of the respective labeled probe of the Universal ProbeLibrary Set, as well as from the human and gene-specific primers (Table 1).

2.10. Quantitative real-time PCR

Quantitative real-time PCR reactions were performed on the Roche LightCycler Instrument 2.0 with LightCycler TaqMan Master (Roche Cat. No. 04 535 286 001). Briefly, 20-μl reaction solutions contained 5 μl generated cDNA template, 4 μl Master Mix, 0.2 μl of 10 μM probe, 0.4 μl of 10 μM forward primer, 0.4 μl of 10 μM reversed primer, and 10 μl water. The q-RT PCR program was conducted at 95 °C for 10 min, 45 cycles of 95 °C for 10 s, 72 °C for 1 s, and 40 °C for 30 s. At the end of the program a melt curve analysis was performed. At the end of each q-RT-PCR run, the data were automatically analyzed, and an amplification plot was generated for each cDNA sample. From each of these plots, the LightCycler4 Data analysis software automatically calculated the CP value (crossing point: the turning point corresponds to the first maximum of the second derivative curve), which indicates the beginning of exponential amplification. The mRNA level was normalized with reference to the amount of the housekeeping gene transcript (glyceraldehyde-3-phosphate dehydrogenase [*GAPDH*] mRNA).

2.11. Reverse transcription-polymerase chain reaction (RT-PCR)

The cDNA obtained was amplified with the primers (Table 1). The reaction was cycled 30 times through 30 s at 95 °C, 30 s at 58 °C, and 45 s at 72 °C. The resulting products were analyzed by electrophoresis on 1.5% agarose gels and stained with ethidium bromide. Specific primers for GAPDH were used as controls [24].

2.12. Immunocytochemistry

Human melanocytes seeded on cover glasses were cultured in medium alone or medium supplemented with arbutin (2.5 mM) or anemonin (50 μM) at 37 °C in a 5% CO_2 incubator. After 24 h, the melanocytes were washed with PBS, and fixed with 4% paraformaldehyde. All of the fixed melanocytes were then blocked with 5% normal horse serum in PBS; they were then treated with rabbit polyclonal anti-TYR (C-19, 1:250), anti-TRP1 (G-17, 1:500), and anti-TRP2 (D-18, 1:500) (Santa Cruz Biotechnology) at room temperature for 90 min. After several washes, the cells were incubated with Cy3-conjugated anti-rabbit IgG in 1:500 dilution (Jackson ImmunoResearch, West Grove, PA, USA) at room temperature for 60 min. After three washes with PBS, the cells that were on slides were covered with anti-fade reagent and visualized using a confocal fluorescent microscope (Bio-Rad MRC-1000).

2.13. Statistical analysis

The nonparametric Mann—Whitney *U*-test was used to compare differences between the groups. Significance was assumed at a probability value of less than or equal to 0.05. Each experiment was repeated at least three times.

3. Results and discussion

3.1. Cell viability after exposure to anemonin from *C. crassifolia*

The active compound, anemonin (Fig. 1), was isolated from the leaves of *C. crassifolia*. The anemonin structure was identified by directly comparing its physical and spectral data (^{1}H NMR and ^{13}C NMR) with the previously reported data [20]. Anemonin has been reported to have antipyretic, sedative [11], and anti-inflammatory activities [17]. In the

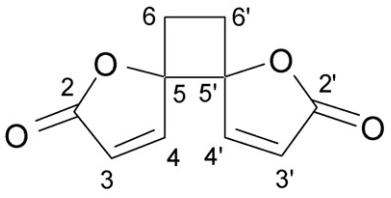

Fig. 1 The structure of anemonin isolated from *Clematis crassifolia*.

present study, human melanocytes were used as the cell model for examining the inhibitory effect of test samples, including anemonin, on TYR and melanin contents. To show that the test samples did not have cytotoxic effects on human melanocytes, an MTT assay using skin melanocytes was done first. Our results showed that the cell viability of melanocytes treated in 50 μM anemonin was 96.4%; this suggests that, after treatment for 24 h, anemonin did not affect cell viability. Thus, given the low cytotoxic effect of anemonin on human melanocytes, the inhibitory effects of anemonin on TYR activity and melanin contents were assessed.

3.2. Anti-TYR activity and melanin content in anemonin-treated melanocytes

The inhibition of TYR during melanin synthesis is a major strategy for developing new whitening agents. Therefore, human melanocytes were used to evaluate the anti-TYR and melanin-decreasing activities of anemonin. The commercial whitening agent, arbutin, was used as the positive control. As shown in Fig. 2A, anemonin inhibited the cellular TYR activity of melanocytes in a concentration-dependent manner within the range of 10—50 μM. The IC_{50} value for anemonin suppression of TYR activity was estimated to be 43.4 μM. In contrast, arbutin given at a concentration of 50 μM resulted in only 9% inhibition. The melanin content of anemonin-treated melanocytes is shown in Fig. 3A. Compared to the control group, treatment with anemonin (20, 40, 50 μM) for 24 h reduced melanin slightly. Following treatment with 50 μM anemonin for 8, 16, 24, or 48 h, time-dependent inhibition of TYR activity (Fig. 2B) was seen, with a significantly reduced melanin content at 48 h (Fig. 3B). These results are in agreement with a previous report showing that melanin synthesis in normal melanocytes is not markedly changed in a 1-day culture; hinokitiol has been reported to reduce the melanin levels in Mel-Ab cells after 3 days of culture [24,25].

3.3. Effects of anemonin on the MITF and TYR genes as well as TRPs proteins expression in human melanocytes

To study the hypopigmentary effect of anemonin, the mechanism of action of anemonin with respect to melanin formation was evaluated, since melanin is one of the heteropolymers that is produced inside melanosomes by the TYR enzyme, which acts on the TYR precursor material found in melanocytes. It has been reported that other factors, such as metal ions and the TRP enzymes (TYR, TRP1, and TRP2/DCT) also affect the production of melanin. These proteins constitute a specific family of membrane proteins that are structurally related but that have distinct enzymatic functions [26]. The effects of anemonin on these proteins after 24 h anemonin treatment were evaluated using Western blotting. Melanocytes were exposed to various concentrations of anemonin (20, 30, 40, and 50 μM); this resulted in dose-dependent decreases in TYR, TRP1, and TRP2/DCT expression (Fig. 4A). When anemonin was used at a concentration of 50 μM,

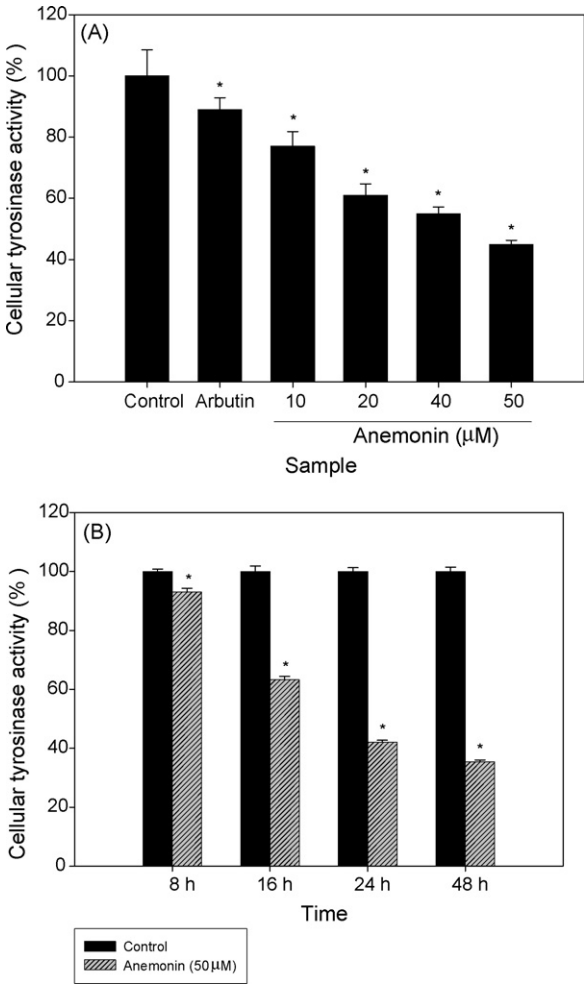

Fig. 2 Cellular TYR activity by anemonin in human melanocytes. Cells (10^5) were cultured for 24 h before being treated with various concentrations of anemonin. After being treated with the individual test samples for specific times, the cell pellets were collected and lysed. After quantifying protein levels, each well of a 96-well plate was plated with lysate (equal amount protein), 2.5 mM L-dopa, and 0.1 M PBS, at pH 6.8. After incubation at 37 °C for 1 h, the absorbance was measured at 475 nm using an enzyme-linked immunosorbent assay reader. (A) Treated with various concentrations of anemonin (10, 20, 40, and 50 μM); (B) treated with anemonin (50 μM) for various time (8, 16, 24, and 48 h). Data were analyzed for statistical significance ($P < 0.05$) by means of the non-parametric Mann—Whitney *U*-test.

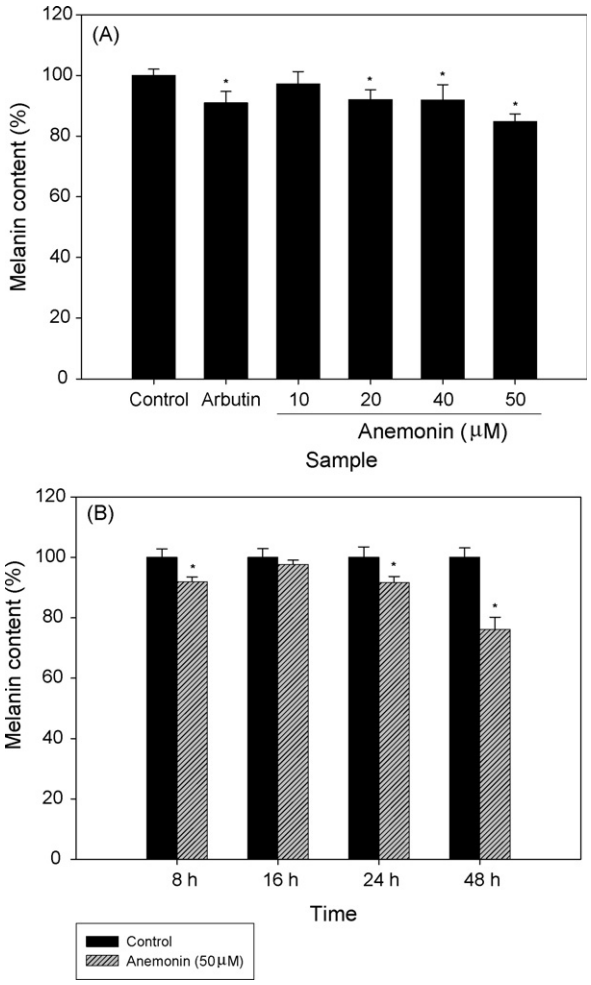

Fig. 3 Melanocyte melanin content after anemonin treatment. Cells were treated with the test samples for specific times. Cell pellets were dissolved in 1 N NaOH at 37 °C overnight. The optical densities of the supernatants were measured at 450 nm using an enzyme-linked immunosorbent assay reader. (A) Treated with various concentrations of anemonin (10, 20, 40, and 50 μM); (B) treated with anemonin (50 μM) for various time (8, 16, 24, and 48 h). Data were analyzed for statistical significance ($P < 0.05$) using the nonparametric Mann—Whitney *U*-test.

the expressions of these 3 proteins decreased over time (Fig. 4B). The reduction in activity with anemonin treatment was compared with that of the control preparations using Quantity One 1-D Analysis Software. The positive control, arbutin (at the IC_{50} value, 2.5 mM), reduced TYR protein activity, but had almost no effect on the other TRPs. Further studies using immunocytochemical staining also supported the fact that anemonin suppressed TYR, TRP1, and TRP2 protein expression (Fig. 4C). Taken together, these observations suggest that anemonin reduced the expression of three TRPs, particularly TYR and TRP2, in a concentration-dependent manner. Arbutin and anemonin reduce melanin synthesis in melanocytes through different mechanisms of action.

Based on the present study, anemonin was found to decrease the levels of the pigment-related proteins TYR and TRP2. Next, in order to determine whether the observed decrease in the expressions of TYR, TRP1, and TRP2/DCT in the anemonin-treated cells was the result of decreased transcription of the *TYR*, *TYRP1*, and *TYRP2* genes, qRT-PCR was used to measure the degree of these proteins' mRNA expression in anemonin-treated melanocytes; *GAPDH* was used as the housekeeping gene. Anemonin down-regulated the levels of mRNAs encoding TYR, TRP1, and TRP2/DCT in a dose-dependent manner. Compared to the untreated control values, at anemonin concentrations of 20, 30, 40 and 50 μM, expression was decreased 0.06-, 0.20-, 0.24-, and 0.29-fold, respectively, for the *TYR* gene, 0.01-, 0.08-, 0.26-, and 0.41-fold, respectively, for the *TYRP1* gene, and 0.34-, 0.57-, 0.73-, and 0.80-fold, respectively, for the *TYRP2* gene (Fig. 5A). Significant down-regulation of *TYRP2* was noted in cultured human melanocytes after the addition of anemonin. MITF is a factor that effectively transactivates the tyrosinase, TRP1, and TRP2 genes; it is considered to be a key regulator of melanocyte development [27]. Therefore, the effect of anemonin on *MITF* expression was evaluated. As shown in Fig. 5B, based on the RT-PCR analysis, the upstream transcription factor *MITF* was down-regulated in a dose-dependent manner.

Melanin is synthesized by a multi-step pathway. Tyrosinase is the key enzyme in the formation of melanin, since it catalyzes the rate-limiting step; TRP1 and TRP2 are also involved in melanin synthesis. TRP1 and TRP2 are transmembrane protein-spanning melanosomal membranes [28]; however, the function of TRP1 in human melanogenesis has not yet been well elucidated. In murine pigment cells, TRP1 has been reported to display TYR-like activity [29]. In addition, in mouse melanocytes, TRP1 has been reported to influence TYR activity by forming a complex and/or stabilizing TYR [30—32]. Nevertheless, in the present study, anemonin did have a slight effect on TRP1, and anemonin substantially reduced the TRP2/DCT content of melanocytes. TRP2 functions as a dopachrome tautomerase downstream of TYR in the melanogenic pathway [33]. It has also been reported to be related to the quantity and the quality of the melanin produced during melanin biosynthesis [34]. In addition to these TYR-related proteins, melanin synthesis is also controlled by other factors, including UV exposure, growth factors, interleukins, prostaglandins, interferons, and hormones [35—37].

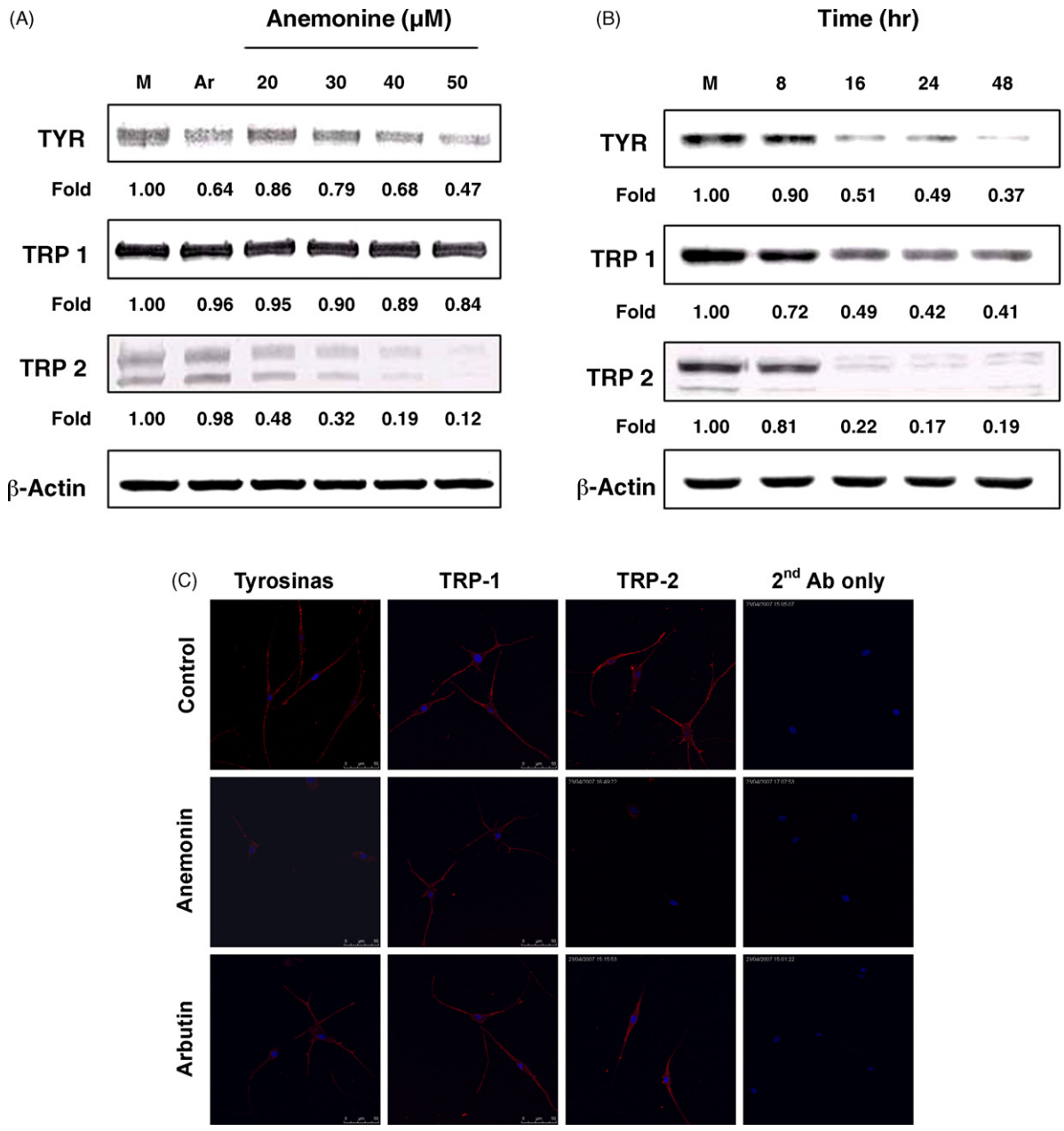

Fig. 4 Expression of TRPs in anemonin- and arbutin-treated human melanocytes. Cells were treated with medium or test compounds for 24 h. Cells were then harvested, and the lysates (10 μg protein) were separated using 10% SDS-PAGE, followed by electroblotting and immunostaining with antibodies to TYR, TRP1, and TRP2/DCT. (A) Treated with various concentrations of anemonin. M, medium only; Ar, 2.5 mM arbutin, the extent of protein loading was evaluated by Western blotting with antibody to β-actin. (B) Treated with anemonin (50 μM) for various time periods (8, 16, 24, and 48 h). The semiquantitative analysis was calculated using Quantity One 1-D Analysis Software. (C) Immunocytochemical staining of melanocytes with antibodies against TYR, TRP1, and TRP2/DCT. Data were analyzed for statistical significance ($P < 0.05$) using the nonparametric Mann—Whitney *U*-test.

The three cloned genes, *TYR*, *TYRP1*, and *TYRP2*, which encode melanosomal proteins, have been grouped together to form the *TYRP* family [38,39] due to their protein sequence homology [40]. The genes encoding these melanogenic enzymes have been cloned and extrinsic factors regulating their expression have recently been identified. It has further been reported that *TYRP1* and *TYRP2* genes may act together to modulate TYR activity [32]. The *TYRP2* gene was also reported to be related to the cytotoxic effects in melanocytes [41]. Anemonin may regulate cytotoxic effects in cultured human melanocytes and, thus, may reduce pigment formation. *MITF* is a known specific transcription factor of the tyrosinase gene family. It is known that the down-regulation of *MITF* may

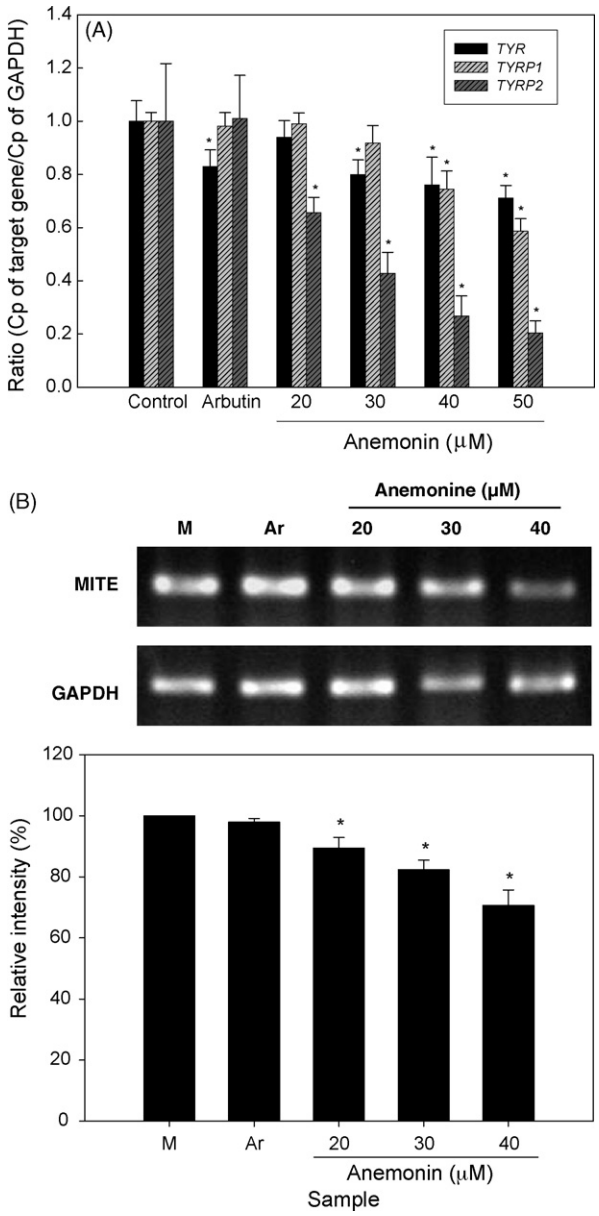

Fig. 5 Expression of (A) TYR-related and (B) *MITF* mRNAs in anemonin- and arbutin-treated human melanocytes. The findings were normalized to the expression of *GAPDH* mRNA. Measurements were conducted in triplicate. The mean expression values for the test samples relative to the mean expression values for negative controls are shown. Control, medium only; arbutin, 2.5 mM. Data were analyzed for statistical significance ($P < 0.05$) using the nonparametric Mann—Whitney *U*-test.

affect the expressions of all tyrosinase genes including *TYR*, *TYRP1*, and *TYRP2* [24]. The present results suggest that *MITF* mRNA levels are reduced by anemonin. The hypopigmentation effect of anemonin may be the result of down-regulation of *MITF* gene expression, which would then repress both the protein and gene expressions of *TYR*, *TRP1*, and *TRP2*.

4. Summary

In the present study, anemonin, a natural compound isolated from *C. crassifolia*, showed potent cellular TYR inhibitory activity in human melanocytes. The results indicate that TYR, TRP1, and TRP2 activity, particularly TYR and TRP2 activity, is reduced by anemonin at the protein level in melanocytes. qRT-PCR was used to determine whether the down-regulation of these proteins occurs at the transcriptional level; *TYR*, *TYRP1*, and *TYRP2* mRNA levels were found to be reduced by anemonin. Therefore, during the process of melanogenesis in melanocytes, anemonin not only inhibits cellular TYR activity but also affects the protein and mRNA levels. Thus, in melanin synthesis, anemonin may regulate both translational and transcriptional levels. Melanogenesis is regulated by a series of enzymes under the control of *MITF*. In the present study, anemonin also reduced *MITF* transcription. Some TYR inhibitors that have been isolated from plants have been found to suppress melanogenesis. Studies have emphasized their use in developing preparations for the prevention and/or treatment of hyperpigmentation. The natural compound, anemonin, may prove to be an effective whitening agent that could be used in skin care cosmetics or as a hypopigmentary agent.

Acknowledgments

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