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ORIGINAL PAPER

Ascorbic acid increases the activity and synthesis of tyrosinase in B16F10 cells through activation of p38 mitogen-activated protein kinase

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Abstract Ascorbic acid, a potential antioxidant, is known to inhibit melanogenesis. However, there are conflicting findings that ascorbic acid has very low stability and acts as a pro-oxidant, eventually increasing proliferation and melanin content in melanoma cells. In the present study, we explored the effects of ascorbic acid on the activity and expression of tyrosinase and melanin pigmentation in the presence and absence of α-melanocyte-stimulating hormone (α-MSH) using B16F10 melanoma cells. The mechanism by which ascorbic acid stimulated the expression of tyrosinase was also investigated. No inhibitory effect on melanin content was observed in ascorbic acid-treated cells, regardless of the presence of α-MSH. Ascorbic acid stimulated the activity and expression of tyrosinase and increased the expression of melanogenic regulatory factors, such as tyrosinase-related protein-1 (TRP-1), dihydroxyphenylalaminechrome tautomerase (TRP-2), and microphthalmia-associated transcription factor (MITF). Ascorbic acid also induced phosphorylation of p38 mitogen-activated protein kinase (MAPK). The inhibition of p38 MAPK pathway by SB203580 led to the suppression of tyrosinase, TRP-1, and TRP-2 expression in cells treated with ascorbic acid. Combined treatment with *N*-acetyl-L-cysteine and/or desferrioxamine mesylate attenuated the stimulating effect of ascorbic acid on tyrosinase activation in the cells. Collectively, ascorbic acid stimulates tyrosinase activity and expression in B16F10 cells via activation of p38 MAPK signaling and subsequent up-regulation of MITF, tyrosinase, and TRP expression.

Keywords Ascorbic acid · Melanogenesis · Tyrosinase · p38 mitogen-activated protein kinase · Oxidative stress

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Introduction

Melanogenesis provides photoprotection of the skin against ultraviolet radiation through the production of a pigmented polymer, melanin, in melanocytes [11, 27]. In contrast, excessive synthesis of melanin may lead to various hyperpigmentation disorders. Melanogenesis consists of many enzymatic oxidation steps and is regulated by several enzymes, such as tyrosinase, tyrosinase-related protein-1 (TRP-1), and dihydroxyphenylalaminechrome tautomerase (TRP-2) [5, 34]. The regulation of melanin synthesis is tightly controlled by mitogen-activated protein kinases

(MAPK), including extracellular signal-regulated kinase (ERK) and p38 MAPK [14, 17]. Microphthalmia-associated transcription factor (MITF) is also a strong stimulator of melanocyte-specific enzymes [18, 28, 34].

Many melanogenic inhibitors have been used in cosmetic and medicinal industries. Arbutin, hydroquinone, kojic acid, retinoids, and vitamins are well-known therapeutic melanogenesis inhibitors. Of these inhibitors, vitamin C (ascorbic acid) is known to have various physiological and pharmacological functions in antioxidation, antiviral, intestinal absorption, and cardiovascular diseases [1, 24]. Ascorbic acid is also used to treat hyperpigmentation, and this effect has been considered to be related to its antioxidant functions [8, 16, 23]. However, ascorbic acid is known to have very low stability and thus, easily decomposes in response to oxidants, metal ions, and ultraviolet light as compared to other vitamins [6, 9]. Ascorbic acid also may act as a pro-oxidant and induce cell death by oxidative stress [26, 32]. Furthermore, it stimulates proliferation and melanin content in cultured B16F10 melanoma cells [13]. These different and opposite effects of ascorbic acid led us to explore whether ascorbic acid is actually capable of inhibiting melanogenesis.

In this study, we examined the effects of ascorbic acid on the activity and expression of tyrosinase and melanin pigmentation in the presence and absence of α-MSH using B16F10 melanoma cells. The mechanisms by which ascorbic acid affects the expression of tyrosinase and TRPs were also investigated.

Materials and methods

Chemicals and laboratory ware

L-Ascorbic acid (A1417; ≥99.0% purify) and p38 MAPK inhibitor, SB203580 were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and TOCRIS (Bristol, UK), respectively. Unless otherwise specified, chemicals and laboratory wares were obtained from Sigma Chemical Co. and SPL Life Sciences (Pochun, South Korea). All reagents used were prepared immediately before use.

Cell culture

B16F10 murine melanoma cells were cultured in 100-mm culture dishes in Dulbecco's minimal essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS; HyClone, Logan, UT, USA) and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin). When the cells reached >95% confluence, the cells were collected and 1 ml or 200 μl cell suspensions (1×10^5 cells/ml) were divided into 6-well or 96-well flat-bottomed plates, respectively. On day 2 of spread, the culture media were replaced with fresh media containing various concentrations (0–500 μM) of ascorbic acid, 1 μM α-MSH, and/or 10 μM SB203580. At various times (0–5 days) of incubation, the effects of ascorbic acid on proliferation, tyrosinase activity, and melanin biosynthesis in the cells were determined.

Measurement of cell viability

Water-soluble tetrazolium salt (WST) was used for the evaluation of cell viability in ascorbic acid-treated B16F10 cells. In brief, cells cultured in 96-multiwell plates were exposed to various concentrations (0–500 μM) of ascorbic acid and further incubated at 37°C for 5 days. WST-8 reagent (10 μl/well) was added to the cultures after washing the cells with PBS and absorbance was measured at 450 nm using a microplate reader (Packard Instrument Co., Downers Grove, IL, USA) after 2 h of incubation.

Assay of cellular tyrosinase activity

B16F10 cells at 70–80% confluence were exposed to various concentrations of ascorbic acid and further incubated for 2 days. The cells were harvested and then incubated for 30 min at 4°C in lysis buffer containing 150 μl of 0.1 M sodium phosphate buffer (pH 6.8), 1% Triton X-100, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The lysates were centrifuged at 12,000×*g* for 30 min. After quantifying protein levels and adjusting concentrations with lysis buffer, the supernatants (50 μl of each lysate) were mixed with 100 μl of 0.1 M sodium phosphate buffer (pH 6.8) and 40 μl of 1.5 mM tyrosine in a 96-well plate. Absorbance was measured at 405 nm at 37°C using an ELISA plate reader (Packard Instrument Co.).

Tyrosinase zymography

Cellular tyrosinase activity was also determined by zymography. Cell lysates were made from ascorbic acid-treated B16F10 cells after 2 days of treatment, as described above. Aliquots of the conditioned lysate containing 50 μg of protein were mixed with loading buffer without *β*-mercaptoethanol and the proteins were separated in 12% polyacrylamide gel containing sodium dodecyl sulfate without a boiling step. After electrophoresis, the gels were equilibrated in 100 mM sodium phosphate buffer (pH 6.8) for 1 h and then incubated in 30 ml of 5 mM L-DOPA until the detection of bands, which correlated with tyrosinase. In this assay system, the band intensity was proportional to the activity of tyrosinase. The intensity of the bands was

estimated using a Gel-Print System (Core Bio Corp., Seoul, South Korea).

Measurement of cellular melanin content

In brief, B16F10 melanoma cells cultured in 6-well plates were treated with ascorbic acid in the presence and absence of 10 μM SB203580 or 1 μM α-MSH for various times (0–5 days). Cells were harvested, washed twice with PBS, and then dried at 60°C for 30 min. The dried samples were incubated in 500 μl of 1 N NaOH for 1 h at 80°C and then centrifuged at 12,000×*g* for 30 min. Optical density of the supernatants was measured at 405 nm using an ELISA plate reader. The amount of melanin content was calculated after comparing it to the standard curves of synthetic melanin and expressed as μg/well of 6-well plates. In addition, the pigmentation of each cell lysate was photographed using a digital camera just before determining the melanin contents.

Western blot analysis

B16F10 melanoma cells were resuspended in a nonidet P (NP)-40 lysis buffer (30 mM Tris–Cl (pH 7.5), 1 mM EDTA, 150 mM NaCl, and 1% NP-40) for 20 min and protein contents of each lysate were quantified using the Bradford method [3]. An equal amount of protein samples (40 μg/sample) were separated by 15% SDS-PAGE and blotted onto PVDF membranes. The blots were probed with primary antibodies and then incubated with a horseradish peroxidase-conjugated anti-IgG in blocking buffer for 1 h. After washing, the blots were developed with enhanced chemiluminescence (ECL) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and exposed to X-ray film (Eastman-Kodak, Rochester, NY, USA). The polyclonal antibodies specific to tyrosinase (sc-15341), TRP-1 (sc-25543), TRP-2 (sc-25544), MITF (sc-25386), ERK (sc-94), and the monoclonal antibodies specific to p-ERK (sc-7383), p-JNK (sc-6254), p-p38 (sc-7973) were purchased from Santa Cruz Biotechnology.

RNA preparation and polymerase chain reaction

Total RNA was isolated from B16 cells according to the manufacturer's instructions (SV Total RNA Isolation System, Promega, Madison, WI, USA). Reverse transcription and polymerase chain reaction (PCR) amplification were performed using an Access RT-PCR System (Promega) according to the manufacturer's protocol. The primer sequences used for PCR were designed according to Kim et al. [19] and were as follows: tyrosinase, 5′-GGC CAG CTT TCA GGC AGA GGT-3′ (forward), 5′-TGG TGC TTC ATG GGC AAA ATC-3′ (reverse); *β*-actin, 5′-CGA GCG GGA AAT CGT GCG TGA CAT TAA GGA GA-3′ (forward), 5′-CGT CAT ACT CCT GCT TGC TGA TCC ACA TCT GC-3′ (reverse). PCR was performed for 26–30 cycles at 94°C for 1 min, 55–58°C for 1 min, and 72°C for 1 min in a DNA thermal cycler (model PTC-100, Waltham, MA, USA). PCR products were analyzed on 1.5% agarose gels and visualized using ethidium bromide staining. Band intensity was calculated using a gel imaging system (model F1-F2 Fuses type T2A, BIO-RAD, Segrate, Italy).

cAMP assay

B16F10 melanoma cells were stimulated with 1 μM α-MSH for 1 h at 37°C in the presence and absence of ascorbic acid. The concentration of cellular cAMP was determined using a cAMP immunoassay kit (Cayman, Ann Arbor, MI, USA) according to the manufacturer's instructions, and was expressed as the percentage to untreated controls.

Statistical analyses

Unless otherwise indicated, all data are expressed as mean ± standard deviation (SD) from three independent or more experiments. One-way ANOVA followed by a Scheffe's test was used for multiple comparisons using SPSS version 18.0 software. $P < 0.05$ was considered statistically significant.

Results

Effects of ascorbic acid on viability and melanin content of B16F10 melanoma cells

When considering the previous studies and the unstable property of ascorbic acid [6, 7, 31], this study used its concentration ranging from 50 to 500 μM. Ascorbic acid at the concentrations used did not cause any toxicity to the cells, whereas 50 and 100 μM concentrations increased viability of the cells significantly ($P < 0.05$) (Fig. 1a). Arbutin and kojic acid at the same concentrations had no effects on cell viability (data not shown). Treatment with ascorbic acid at the concentration of 50–200 μM increased the melanin content in these cells (Fig. 1b).

Stimulating effects of ascorbic acid on the activity and expression of tyrosinase in B16F10 melanoma cells

The addition of ascorbic acid to the cells induced a significant stimulation on the activity of tyrosinase (Fig. 2a). This finding was similar to the results from tyrosinase

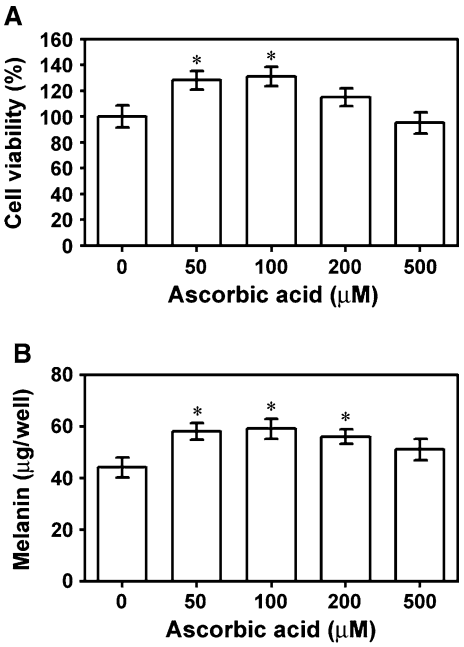

Fig. 1 Effects of ascorbic acid on viability and melanin content in B16F10 cells. Cells cultured in 96-multiwell plates or 6-well plates were exposed to the indicated doses (0–500 μM) of ascorbic acid for 5 days and then processed for the analysis of viability (**a**) and melanin content (**b**), respectively. $*P < 0.05$ versus the untreated control values

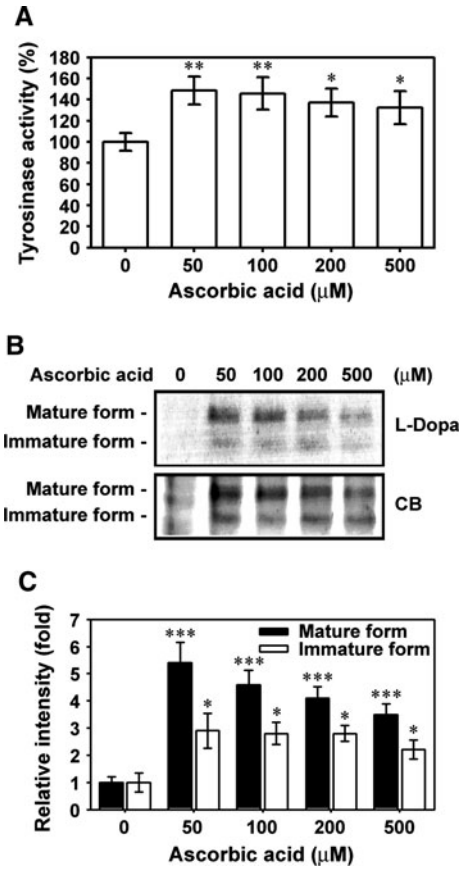

Fig. 2 Effect of ascorbic acid on tyrosinase activity in B16F10 melanoma cells. Cells were cultured with the indicated concentrations (0–500 μM) of ascorbic acid for 2 days and cellular tyrosinase activity was measured by an ELISA plate reader (**a**) or zymography (**b**), as described in "Materials and methods". **c** The relative intensities (fold) of mature and immature forms were determined from triplicate experiments. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ versus untreated control values. *CB* Coomassie blue

zymography; both the mature and immature forms of tyrosinase were increased by treating the cells with ascorbic acid (Fig. 2b). The band intensity of the mature form with the addition of 50 and 100 μM ascorbic acid was augmented by 5.4- and 4.6-fold, respectively, as compared to untreated control cells (Fig. 2c). The data from western blot and RT-PCR assays also showed the increase in tyrosinase at the protein and mRNA levels in B16F10 cells (Fig. 3a, b). These results suggested that ascorbic acid up-regulates cellular enzymatic activity of tyrosinase in melanoma cells as well as increases mRNA and protein levels of the enzyme.

Up-regulation of TRPs and MITF in ascorbic acid-treated B16F10 melanoma cells

Similar to tyrosinase, the level of TRP-1 protein was significantly augmented by ascorbic acid at all the concentrations added, while TRP-2 increased at 50 and 100 μM (Fig. 4a, b). Subsequent experiments revealed that ascorbic acid stimulates the expression of MITF protein (Fig. 4c). For example, ascorbic acid at 50 μM increased MITF levels up to 3.6-fold ($P < 0.01$) as compared to the untreated control and this increase was also seen when cells were exposed to 500 μM ascorbic acid (Fig. 4d).

Regulatory effects of p38 MAPK on tyrosinase and TRP expression, and melanin content in ascorbic acid-stimulated melanoma cells

ERK signaling is known to regulate melanogenesis in two ways: inhibition of ERK pathway increases tyrosinase activity, while sustained ERK activation reduces melanin synthesis through MITF degradation and subsequent down-regulation of tyrosinase and TRP-1 [18]. In contrast, the activation of p38 MAPK is reported to increase melanin synthesis by stimulating MITF expression and tyrosinase transcription [14]. We subsequently analyzed the pattern of MAPK phosphorylation in ascorbic acid-treated melanoma cells. As shown in Fig. 5, the level of p-ERK was not changed by ascorbic acid at a significant level, whereas the level of p-p38 increased after ascorbic acid treatment.

We further determined the roles of p38 MAPK signaling on the expression of tyrosinase and TRPs in ascorbic

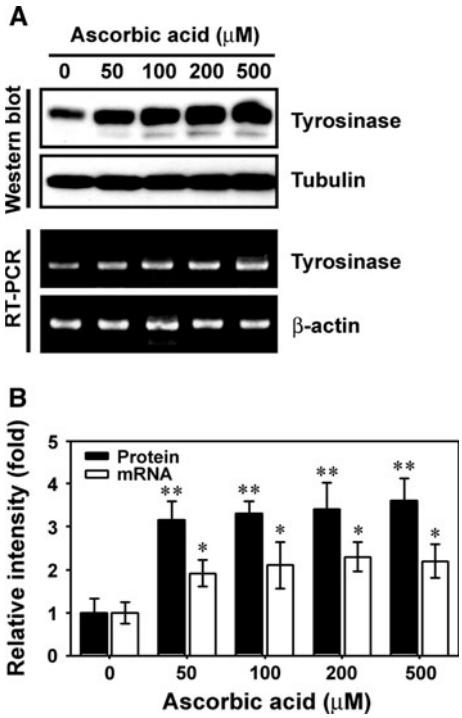

Fig. 3 Effect of ascorbic acid on the expression of tyrosinase in B16F10 melanoma cells. **a** Cells were cultured with increasing doses (0–500 μM) of ascorbic acid for 2 days. Protein (*upper panel*) and mRNA levels (*lower panel*) of tyrosinase were determined by western blotting and RT-PCR, respectively. **b** The relative intensities of tyrosinase protein and mRNA levels were determined from three independent experiments. α-Tubulin and *β*-actin primers were used as controls to ensure the equal loading of proteins and target cDNA, respectively. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus untreated control values

acid-exposed B16F10 cells. Pretreatment of cells with p38 MAPK inhibitor (10 μM SB203580) significantly attenuated the protein levels of tyrosinase, TRP-1, and TRP-2 those had been stimulated with ascorbic acid (Fig. 6a, b). In addition, ascorbic acid-mediated increase in melanin content was reduced by treating cells with 10 μM SB203580 (Fig. 6c).

Ascorbic acid did not affect α-MSH-stimulated melanin pigmentation in B16F10 melanoma cells

As shown in Fig. 7a, a naked-eye view of cell pellets indicated that α-MSH-stimulated melanin pigmentation in melanoma cells, but ascorbic acid did not reduce the accumulation of melanin. This result was supported by the absorbance analysis at 405 nm using an ELISA reader, where ascorbic acid did not reduce cellular melanin content in the cells (Fig. 7b).

No effect of ascorbic acid on α-MSH-mediated melanogenesis was supported by the observation of cellular tyrosinase activity (Fig. 8a). Ascorbic acid also did not suppress the α-MSH-mediated increase in cAMP levels in B16F10 cells (Fig. 8b).

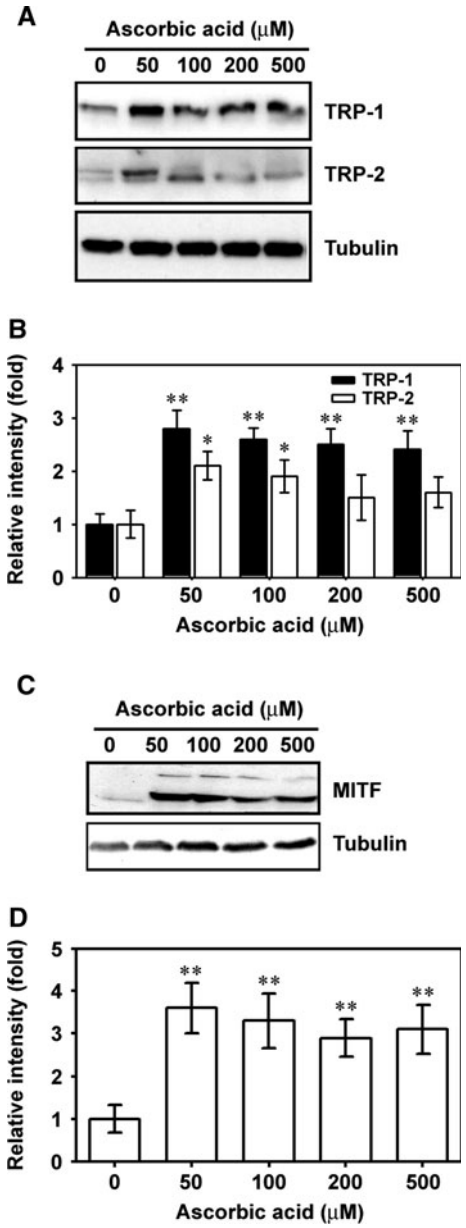

Fig. 4 Effects of ascorbic acid on the protein levels of TRP-1, TRP-2, and MITF in B16F10 cells. Cells were treated with the indicated concentrations of ascorbic acid for 2 days and then the protein expression of TRPs (**a**) and MITF (**c**) was determined by immunoblotting. The relative intensities (fold) of these proteins (**b**, **d**) were calculated from triplicate experiments by comparing that of tubulin. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus untreated control values

Combined incubation with antioxidant or metal-ion chelator inhibit ascorbic acid-mediated stimulation of tyrosinase activity in B16F10 cells

B16F10 cells were incubated with 500 μM ascorbic acid in the presence and absence of 5 mM *N*-acetyl-L-cysteine (NAC) and/or 5 mM desferrioxamine (DFX) for 48 h and then processed for the determination of cellular tyrosinase

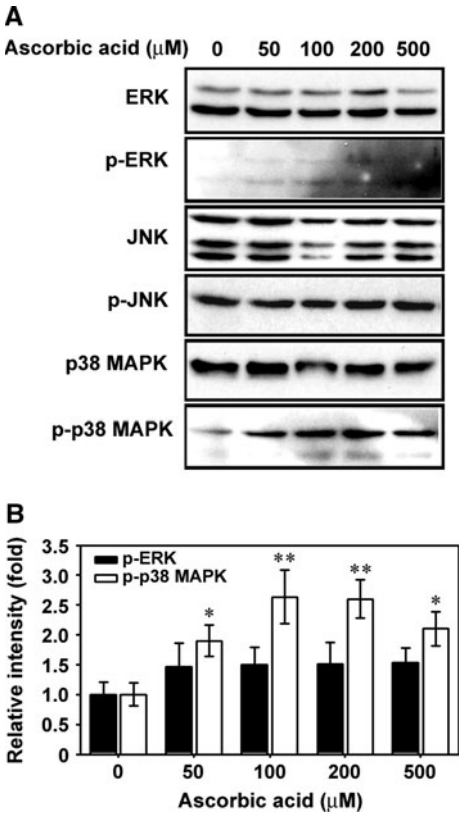

Fig. 5 Effects of ascorbic acid on the activation of MAPK in B16F10 cells. **a** Cells were exposed to increasing doses (0–500 μM) of ascorbic acid for 6 h and cell lysates were subjected to western blot analysis with antibodies against MAPKs. Protein loading was checked by reactions with phosphorylation-independent MAPK antibodies to ensure equal loading. **b** The relative intensities (fold) of p-ERK and p38 MAPK were calculated from triplicate experiments. $*P < 0.05$ and $**P < 0.01$ versus untreated control values

activity (Fig. 9). Co-treatment with NAC and/or DFX significantly reduced ($P < 0.05$) ascorbic acid-mediated stimulation of tyrosinase activity to the basal level. NAC or DFX itself did not affect the activity of tyrosinase in the cells. Similarly, NAC and DFX suppressed ascorbic acid-induced stimulation of tyrosinase expression (data not shown).

Discussion

Numerous investigations are currently focused on the development of new therapeutic agents to prevent hyperpigmentation. It is well known that ascorbic acid works as an antioxidant to inhibit melanogenesis. Ascorbic acid also stimulates the biosynthesis of collagen in human dermal fibroblasts and protects skin cells against oxidative stress [25, 29]. However, ascorbic acid cannot penetrate the outer layer of skin because ascorbic acid is water soluble. Ascorbic acid is easily oxidized to dehydro-L-ascorbic acid by air in aqueous solutions and has very low stability against light and heat [9, 30]. This leads to limitations in the application of ascorbic acid in cosmetic and medicinal materials. It was also reported that 0.1 mg of L-ascorbic acid is almost completely decomposed within 3 day when it was dissolved in distilled water at room temperature [6]. In addition, several studies have failed to demonstrate anti-melanogenesis effects of ascorbic acid alone [10, 22]. Further, a recent report shows the increase in melanin content and proliferation rate in cultured B16 melanoma cells exposed to ascorbic acid [13].

In the present study, we highlighted the effects of ascorbic acid on melanogenesis in B16F10 murine melanoma cells in the presence and absence of α-MSH. No inhibitory effect on melanin content was observed in ascorbic acid-treated cells, regardless of supplementation with α-MSH. Ascorbic acid stimulated the activity of tyrosinase and increased the expression of tyrosinase as well as TRPs and MITF in the cells. These stimulating effects were not changed even if the culture media were replaced to a fresh one containing the same dose of ascorbic acid per 2 days (data not shown). This suggests that the unstable chemical property of ascorbic acid does not cause a side effect on the present results at least during the experimental periods used in this study. We also showed that ascorbic acid at the doses, 50 and 100 μM, stimulated viability of the cells. This result is quite similar to the previous findings, where ascorbic acid at 100 μM increased significantly melanin content and proliferation in B16F10 melanoma cells [13]. However, there were opposite findings showing that ascorbic acid induced apoptosis in these cells by acting as a pro-oxidant [7, 17]. We consider that this difference is closely associated with the concentrations of ascorbic acid exposed to cells. For example, ascorbic acid caused a significant toxicity in melanoma cells when it was added to the cultures at approximately 800 μM for 24 h [7] or at 10 mM for 2 h [17]. Moreover, it is important to consider that ascorbic acid at relatively high concentrations (mM) is needed for its physiological functions in vivo.

MAPKs are involved in the control of melanogenesis in melanocytes. Commonly, p38 MAPK signaling activates MITF [2, 33], whereas the ERK signaling pathway down-regulates MITF [18]. Thus, the activation of p38 MAPK or the blockage of ERK cascade signaling suggests the activation of tyrosinase with the attendant accumulation of melanin [12, 14]. The present study shows that ascorbic acid induced the phosphorylation of p38 MAPK, whereas no effect was observed in levels of p-ERK. It was also found that the inhibition of the p38 MAPK pathway led to the reduction of ascorbic acid-mediated expression of tyrosinase, TRP-1, and TRP-2 proteins in the cells. This supported the hypothesis that ascorbic acid-mediated increase in melanin content was

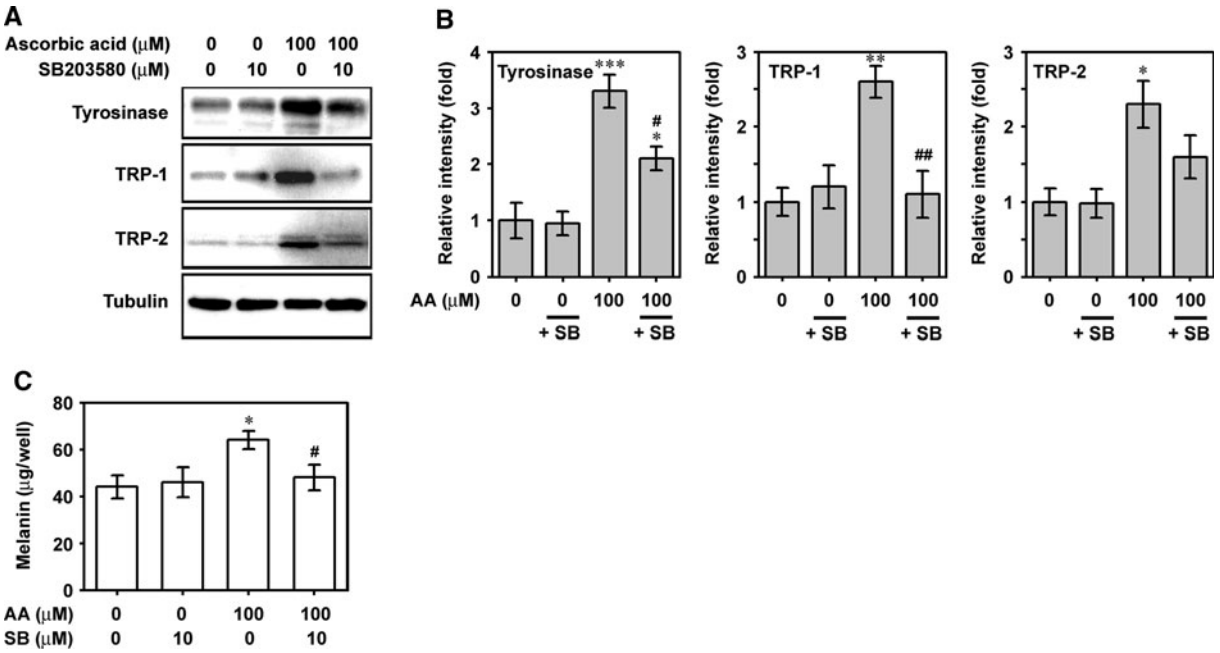

Fig. 6 Regulating effects of p38 MAPK inhibitor, SB203580, on ascorbic acid-mediated stimulation of tyrosinase and TRPs expression, and melanin pigmentation in B16F10 cells. **a** Cells were exposed to 100 μM ascorbic acid in the presence and absence of a specific p38 MAPK inhibitor, SB203580 (10 μM), for 2 days and cell lysates were processed for the analyses of tyrosinase and TRPs by western blotting. **b** Tubulin antibody was used a loading control and relative intensities (fold) of the proteins from triplicate experiments were shown. **c** Cells were also treated with ascorbic acid in the presence and absence of 10 μM SB203580 for 5 days and then processed to determine melanin content. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus untreated control values. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus ascorbic acid treatment alone

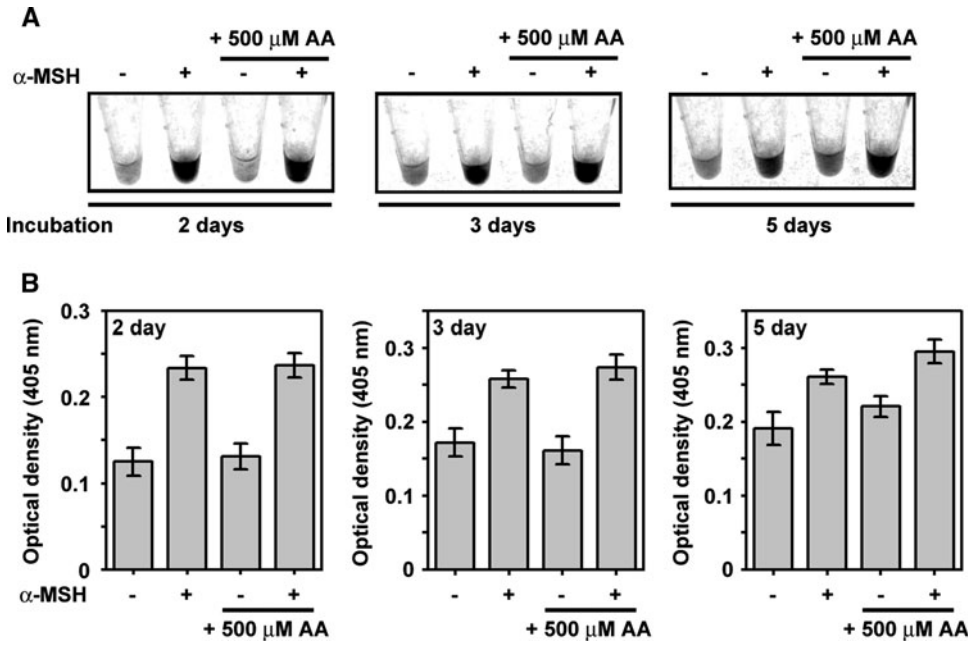

Fig. 7 Effect of ascorbic acid on melanin content in α-MSH-stimulated B16F10 melanoma cells. Cells were cultured in 6-well plates with 500 μM ascorbic acid in the presence and absence of 1 μM α-MSH for various times (2–5 days). **a** Cells were collected into new tubes and the pellets were photographed. **b** Cell pellets were resuspended in a NaOH solution and absorbance of the supernatants was measured at 405 nm

correlated with up-regulation of tyrosinase and TRPs and this was controlled by the p38 MAPK signaling pathway.

α-MSH stimulates tyrosinase and melanin pigmentation via activation of adenylyl cyclase and subsequent increase in intracellular cAMP levels [4, 20]. We found that ascorbic acid did not inhibit α-MSH-stimulated melanin biosynthesis in B16F10 melanoma cells. This is not consistent with the results of a previous report showing the reduction of melanin content by ascorbic acid in α-MSH-stimulated B16F10 melanoma cells [7]. No

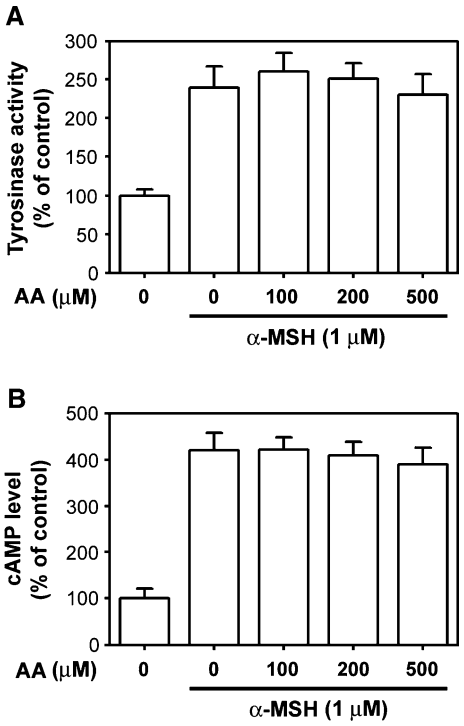

Fig. 8 Effects of ascorbic acid on α-MSH-stimulated tyrosinase activity and cAMP induction in B16F10 melanoma cells. **a** Cells were stimulated with 1 μM α-MSH in the presence of the indicated concentrations of ascorbic acid for 2 days and then processed for the determination of tyrosinase activity. **b** Cellular cAMP level was determined 1 h after the treatment with the indicated doses of ascorbic acid and/or α-MSH

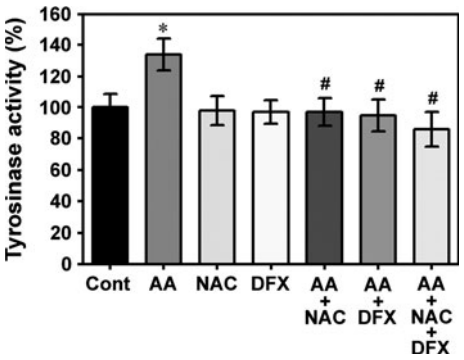

Fig. 9 Effects of NAC and DFX on ascorbic acid-stimulated tyrosinase activity in B16F10 melanoma cells. Cells were exposed to 500 μM ascorbic acid in the presence and absence of 5 mM NAC and 5 mM DFX for 2 days and tyrosinase activity was then determined. $^{*}P < 0.05$ versus untreated control values. $^{\#}P < 0.05$ versus ascorbic acid treatment alone

suppression of tyrosinase activity and cAMP levels by ascorbic acid was also found in α-MSH-stimulated melanoma cells. In contrast, combination with NAC and/or DFX significantly inhibited the stimulating effect of ascorbic acid on tyrosinase activity. Ascorbic acid-stimulated induction of tyrosinase and TRP-1 proteins were also reduced by the combined treatment with NAC or DFX (data not shown). One previous report showed that simultaneous administration of ascorbic acid, L-cysteine, and vitamin E effectively decreases melanin content and inhibits tyrosinase activity in B16 melanoma cells [10]. It was also reported that multivitamin complexes, which are composed of vitamins A, D, E, B1, B2, B5, B6, and C, reduce melanin content more than ascorbic acid itself does [7]. These reports suggest that ascorbic acid alone has a limitation to inhibit melanogenesis.

Several findings have shown that oxidative stress occurs in ascorbic acid-exposed melanoma cells and this is related to its anticancer effect. Ascorbic acid and multivitamins containing ascorbic acid are known to induce cytotoxicity at high concentrations and this is believed to be related to its pro-oxidant effect rather than a direct production of ROS [26, 32]. It was reported that the induction of a pro-oxidant state by ascorbic acid and a subsequent reduction in mitochondrial membrane potential are involved in the caspase-independent apoptotic pathway of B16F10 melanoma cells [17]. When considering the redox cycling activity and these previous findings, it is hypothesized that ascorbic acid-mediated oxidative stress is derived from its pro-oxidant effect. Moreover, a direct exposure to H_2O_2 inhibits melanogenesis in B16 murine melanoma cells, as well as in two human melanoma cell lines [15]. This supports the hypothesis that ascorbic acid may affect melanogenesis by acting as a pro-oxidant rather than by producing free radicals. In B16F10 cells, this property of ascorbic acid might lead to the stimulation of tyrosinase activity and expression. Further, multivitamin complexes have anti-melanogenic effects stronger than ascorbic acid alone, although ascorbic acid is more potent than multivitamins in terms of antioxidant activity [7]. These led us to postulate that the antioxidant activity of a compound is not directly correlated with its ability to inhibit melanogenesis. However, further detailed experiments will be needed to clarify the relationship between the antioxidant activity and anti-melanogenic potential. Furthermore, it is important to consider that the key signaling molecules regulating cellular events are sensitive to intracellular redox state and in vivo the pro-oxidant effect of ascorbic acid can be effectively disappeared by various antioxidants as well as by metal ion chelators [21].

In summary, the present study suggests that ascorbic acid stimulates the activity and synthesis of tyrosinase with attendant increase in melanin content in B16F10 murine melanoma cells. This stimulation was mediated by the p38 MAPK signaling pathway. Combined treatment with NAC and/or DFX inhibited ascorbic acid-stimulated tyrosinase activity in cells. Our present findings strongly suggest that ascorbic acid alone stimulates tyrosinase activity and melanin pigmentation in melanin cells, and does not inhibit

α-MSH-stimulated melanogenesis. Although more detailed experiments using various types of melanoma cells are warranted, and the in vivo regulatory role of ascorbic acid on melanogenesis is still unclear, caution may be indicated regarding the use of ascorbic acid alone for skin whitening.

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