—Regular Article—

Mechanism of Pigmentation by Minocycline in Murine B16 Melanoma Cells

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Long-term treatment with minocycline is known to induce pigmentation or discoloration in tissues but how remains unclear. We investigated the mechanism of pigmentation using B16 melanoma cells. First, we confirmed that intracellular melanin levels increased on minocycline treatment. Then, using the reverse transcriptase-polymerase chain reaction (RT-PCR), we found the expression of mRNA of tyrosinase, tyrosinase-related protein (TRP)-1 and TRP-2, to also be significantly increased by treatment with minocycline at 5 μg/ml for 72 h. These results suggest that the minocycline-induced stimulation of melanogenesis occurs at the transcriptional level. Western-blotting revealed slight phosphorylation of extracellular signal-regulated kinase (ERK) 30–60 min after the minocycline treatment. The mitogen-activated protein kinase kinase 1/2 (MEK1/2) inhibitor U0126 and the p38 inhibitor SB203580 were used to examine the signaling pathway associated with the mRNA expression of tyrosinase, TRP-1, or TRP-2 when B16 melanoma cells were treated with minocycline. The SB203580 inhibited the mRNA expression of tyrosinase and TRP-1, suggesting the minocycline-induced melanogensis occurred *via* a p38 signaling pathway.

Key words——minocycline; pigmentation; tyrosinase; TRP1/2; ERK1/2; melanogenesis

INTRODUCTION

Minocycline, introduced as a broad-spectrum semi-synthetic derivative of tetracycline in 1967,[1] has been used for the treatment of acne vulgaris,[2,3] rheumatoid arthritis,[4] and various other inflammatory diseases.[5,6] Widespread use for acne began in the late 1970s and cutaneos hyperpigmentation has been reported as an adverse effect.[7,8] Minocycline is considered relatively safe because adverse reactions are uncommon and mostly mild, therefore it can be used for long periods at high doses.[9] As minocycline is lipid soluble and highly bound to plasma-protein, it easily penetrates body fluids and distributes in tissues.[10] There are many reports of minocycline-induced hyperpigmentation in skin, subcutaneous fat, nails, teeth, gingivae, oral mucosa, lips, sclerae, heart valves, thyroid, prostate, and lymph nodes *etc.*[11–20] It has been shown that minocycline-induced hyperpigmentation is categorized into several types.[21] Minocycline may cause pigmentation if the breakdown product of the drug itself is pigmented, or if the drug or its metabolite chelates to iron and/or induce melanin deposition. Okada *et al.* reported that iron and minocycline were detected in the pigmented skin associated with minocycline therapy.[22] The pigmentation appears to involve melanin, hemosiderin, or a combination of the two with a possible melanin-drug complex.[23] The mechanisms of minocycline-induced pigmentation, however, have been unclear.

Melanin is synthesized by specialized dendritic cells known as melanocytes in skin, hair, eyes and other areas. Melanogenesis is a characteristic of melanocyte differentiation,[24] which is important in the development and progression of melanomas.[25] Melanin biosynthesis is catalyzed by three melanocyte-specific enzymes: tyrosinase, tyrosinase-related protein (TRP)-1 and TRP-2.[26] Tyrosinase is the rate-limiting enzyme in melanin biosynthesis and catalyzes the hydroxylation of tyrosine to form 3,4-dihydroxyphenylalanine (DOPA) and oxidation of DOPA to produce DOPA-quinone.[27] Therefore, inhibitors of tyrosinase have been used in cosmetics as skin-whitening agents.[28] TRP-2 acts as a DOPAchrometautomerase and catalyzes the rearrangement of DOPAchrome to form 5,6-dihydroxyindole-2-carboxylic acid (DHICA),[29] and TRP-1 oxidizes DHICA to produce carboxylated indole-quinone.[30] Thus,

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TRP-1 and TRP-2 also function in the biosynthesis of melanin downstream of tyrosinase. The expression of these enzymes is strongly regulated by microphthalmia-associated transcription factor (MITF).[31,32] MITF is a the substrate regulated by phosphorylated extra cellular signal-regulated kinase (ERK1/2) and involved in the diferrentiation, proliferation, and survival of melanocytes.[33,34] ERK1/2, part of the Ras/Raf/Mek/Erk mitogen-activated protein kinase (MAPK) signaling pathway, regulates cell survival, proliferation, and differentiation in response to extracellular stimuli.[35] Inhibition of the ERK pathway has been reported to enhance tyrosinase promoter activity, therefore the ERK pathway is closely associated with MITF-mediated melanogenesis.[36]

In this study, we examined the effects of minocycline on melanogenesis in cultured B16 melanoma cells, and whether the minocycline-induced pigmentation was associated with the activation of tyrosinase, TRP1/2, or MITF, which is involved in the ERK pathway. The findings will help in elucidating the mechanism of minocycline-induced pigmentation.

MATERIALS AND METHODS

Reagents For Western blotting, the primary antibodies used were an anti-MITF antibody (ab13703) (Abcam, Cambridge, UK), anti-tyrosinase antibody (ab52493) (Abcam), anti-rat ERK1/2 antibody (Cell Signaling Technology, Inc., Beverly, MA, U.S.A), and anti-phosphorylated-ERK1/2 antibody (Cell Signaling Technology, Inc.). The secondary antibodies used were an anti-rabbit immunoglobulin G (IgG) horse radish peroxidase (HRP)-linked antibody (#7074) (Cell Signaling Technology, Inc.) and anti-mouse IgG-HRP (sc-2005) (Santa Cruz Biotechnology, Santa Cruz, CA). To normalize the total amount of protein, an anti-β-actin antibody (Santa Cruz Biotechnology) was used. Minocycline hydrochloride was purchased from Sigma-Aldrich (St Louis, MO).

Cell Culture Mouse B16 melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Wako Pure Chemicals Co. Ltd., Osaka, Japan) containing 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin G, and 100 mg/ml streptomycin with 5% CO_2 at 37°C. For experiments, cells were seeded on a 6-well plate, or a 60-mm dish.

Viability of B16 Melanoma Cells The effect of minocycline on the proliferation of B16 melanoma cells was determined with the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay. B16 melanoma cells were seeded in a 48-well plate at a density of 5×10^3 cells per well and allowed to attach for 24 h. Then the cells were incubated in fresh medium containing various concentrations of minocycline for 72 h. After the incubation, the MTT solution was added to each well at a final concentration of 0.05 %. After further incubation for 4 h, stop solution (10% sodium dodecyl sulfate (SDS) in 0.1% HCl) was added to each well and incubation was continued for another 24 h. Finally, the cell viability was estimated in terms of absorbance at 590 nm.

Determination of Melanin Content B16 melanoma cells were seeded in a 6-well plate at a density of 2.5×10^4 cells per well and allowed to attach for 24 h. Then the cells were incubated in fresh medium containing 0, 1, 5, 10, 20 and 50 μg/ml per day of minocycline, and the same procedure was repeated once daily for 72 h. For the determination of melanin content, the cells were collected after treatment with a 0.2% ethylenediaminetetraacetic acid (EDTA) solution and washed with phosphate-buffered saline (PBS). After centrifugation, the cell pellets were solubilized in 100 μl of 1 M NaOH at 80°C for 1 h. The melanin content was estimated from the absorbance at 405 nm, and values were corrected for the amount of protein.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total RNA was isolated from B16 cells using a Fast Pure RNA kit (Takara Bio, Shiga, Japan). The first-strand cDNA was synthesized from 2 μg of total RNA with PrimeScript Reverse Transcriptase (Takara Bio). The sequences of specific primers used were as follows; for tyrosinase sense, 5′-ACAGAGGAGAACATCTGCCAGCTT-3′, and tyrosinase antisense, 5′-TGGTGACTCAAC- AGGT GTGAAGGT-3′, for TRP-1 sense, 5′-ATACTGGG-ACCAGATGGCAACACA-3′, and TRP-1 antisense, 5′-ATTGGTCCACCCTCAGTGCTGTTA-3′, and for TRP-2 sense, 5′-AGACTACGTGATCACCAC-GCAACA-3′, and TRP-2 antisense, 5′-TTCCGAC-TAATCAGCGTTGGGTCA-3′. Specific primers for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Clontech) were added as a control. PCR was carried out by incubating each cDNA sample with the primers (0.5 μM each), Blend Taq polymerase (1.25 U: Toyobo, Osaka, Japan), and a deoxynucleotide mix (0.2 mM each: Toyobo). Amplification was car-

ried out for 35 cycles (95℃ for 30 s, annealing at 60℃ for 30 s, and 72℃ for 1 min). The products were then subjected to 2% agarose gel electrophoresis. Bands were stained with ethidium bromide (Sigma) and photographed.

Western Blot Analysis To examine the phosphorylation of ERK1/2, B16 melanoma cells were seeded in 60-mm dishes at a density of 5×10^4 cells per dish, treated with 5 μg/ml of minocycline, and subjected to Western blotting at 5 time points (15, 30, 60, 90, 120 and 180 min) after the treatment. In brief, the cells were washed with cold PBS twice and lysed in cold lysis buffer (10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)-NaOH pH 7.4, 1 % Triton X-100, 5 mM 2Na-EDTA, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, and 1 mM sodium o-vanadate) containing a protease inhibitor cocktail. Proteins (30 μg per lane) were separated by 10% sodium dodecyl sulfate poly-acrylamide gel electrophresis (SDS-PAGE) and transferred to a polyvinylidene fluoride (PVDF) membrane. The membrane was blocked with 1% BSA in Tris-HCl buffer saline-0.1% tween 20 (TBS-T) buffer. ERK and phosphorylated ERK (pERK) were detected with the anti-ERK1/2 antibody and anti-pERK1/2 antibody, respectively. The PVDF membrane was further incubated with the anti-rabbit IgG HRP-conjugated antibody. The blots were further washed with TBS-T buffer, and specific proteins were visualized by using ECL Western blotting detection reagents (GE Healthcare, Piscataway, NJ).

Statistical Analysis Data are presented as the mean±S.E. The significance of differences was evaluated with ANOVAs and p values of less than 0.05 were considered statistically significant.

RESULTS

Cytotoxicity of Minocycline to B16 Melanoma Cells Ths effect of minocycline on the viability of B16 melanoma cells was examined using the MTT assay. The cells were treated with various concentrations of minocycline (1, 5, 10, 20 and 50 μg/ml). As shown in Fig.1, minocycline exhibited no cytotoxicity at 1 μg/ml, but was significantly toxic of concentrations of more than 5 μg/ml ($p<0.05$).

Effect of Minocycline on Melanin Content in B16 Melanoma Cells The effect of minocycline on intracellular melanin content was examined at around 5 μg/ml, a dose which caused slight cytotoxicity. The amount of melanin was significantly increased by minocycline, compared with the control group as shown in Fig. 2 (A) and (B). To investigate whether the melanogenesis is activated *via* a direct effect on tyrosinase, tyrosinase activity was examined in a cell-free system using mushroom tyrosinase. No significant changes were observed in tyrosinase activity at doses of between 1 μg/ml and 50 μg/ml of minocycline (data not shown).

Effect of Minocycline Treatment on mRNA Levels The cells were treated with 0, 1, 5, or 10 μg/ml of minocycline for 72 h and total cellular RNA was extracted. The adequate time point of extraction was

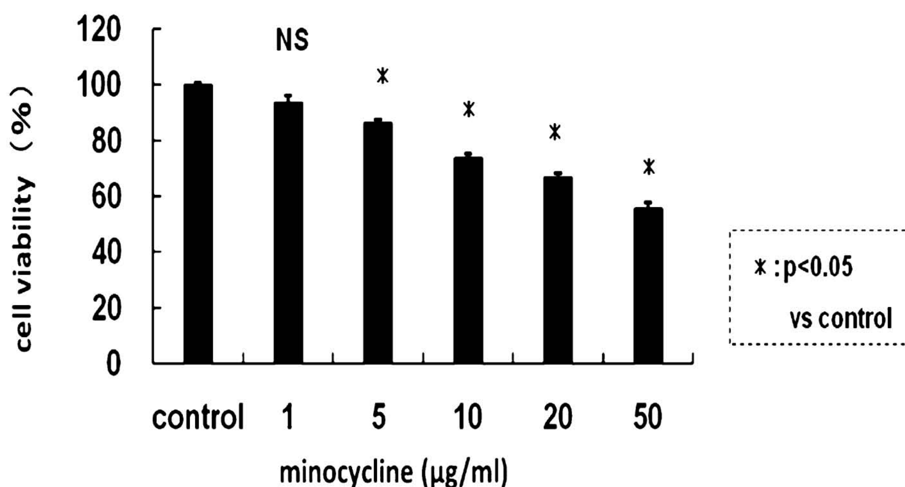

Fig. 1. Cytotoxicity of Minocycline to B16 Melanoma Cells

B16 melanoma cells were incubated with different concentrations of minocycline (1, 5, 10, 20 and 50 μg/ml) for 72 h and collected for the MTT assay. The data represent the mean±S.E. for 3 independent experiments. *$p<0.05$ indicates a significant difference compared with the control.

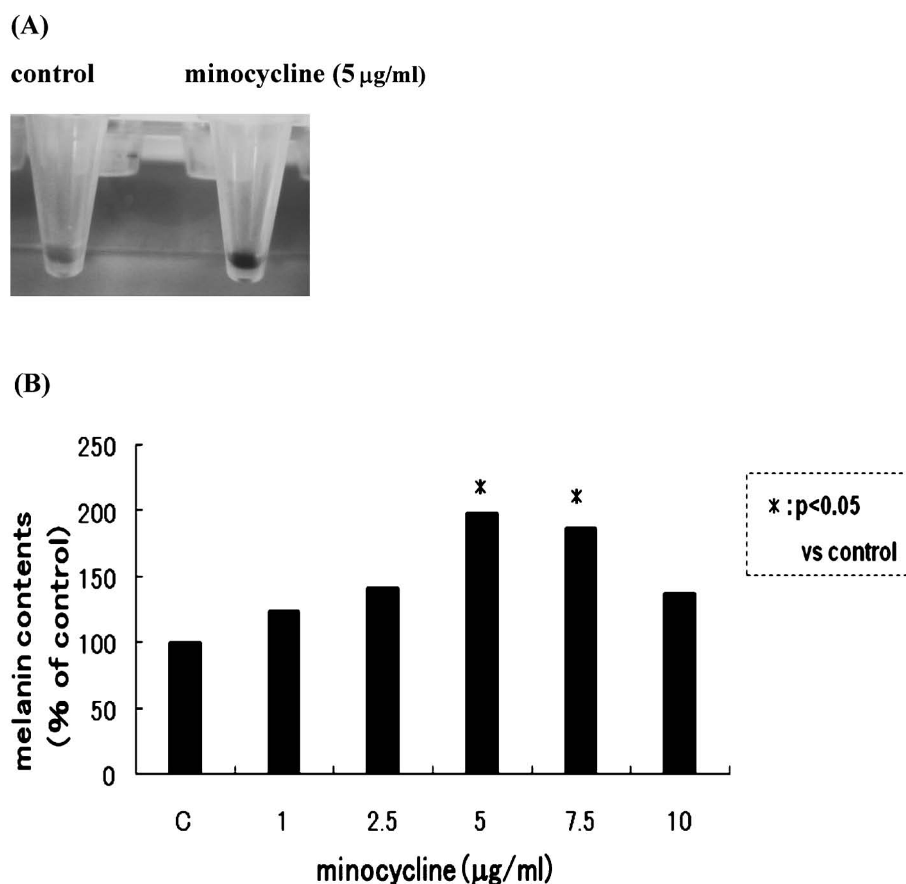

Fig. 2. Effect of Minocycline on Melanin Content in B16 Melanoma Cells

(A) Photograph of precipitated B16 melanoma cells. Cells were incubated for 72 h with and without 5 μg/ml minocycline. (B) B16 melanoma cells were incubated with different concentrations of minocycline (1, 2.5, 5, 7.5 and 10 μg/ml). Melanin content was calculated from the absorbance at 405 nm. Each value is the mean ± S.E. of 3 independent experiments. $*p < 0.05$ indicates a significant difference compared with the control.

determined by preliminary experiment. The mRNA was amplified after reverse transcription with PCR using primers specific for tyrosinase, TRP-1, and TRP-2. As shown in Fig. 3, the mRNA expression of tyrosinase was increased after treatment with 5 μg/ml or more of minocycline for 72 h. As for TRP-1 and TRP-2, the expression of each mRNA was similarly increased after treatment with 5 μg/ml or more of minocycline. These results suggest that the minocycline-induced stimulation of melanogenesis occurs at the transcriptional level.

Effect of a MEK1/2 Inhibitor or p38 Inhibitor on the mRNA Expression of Tyrosinase, TRP-1, and TRP-2 in Minocycline-treated B16 Melanoma Cells

We investigated whether the phosphorylation of ERK is prevented by U0126, a selective inhibitor of MEK (MAPK/ERK) which is the upstream activator of ERK. No inhibition of the mRNA expression of tyrosinase, TRP-1, and TRP-2 was observed when the cells were incubated with U0126 for 1 h before the

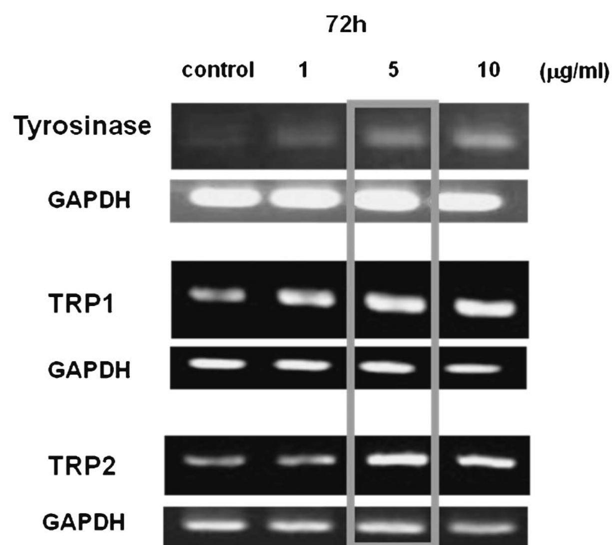

Fig. 3. Effects of Minocycline on mRNA Expression of Tyrosinase, TRP-1 and TRP-2

B16 melanoma cells were incubated with minocycline for 72 h. RNA samples from the treated cells were amplified after reverse transcription with PCR using specific primers for tyrosinase, TRP-1, and TRP-2. PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide.

minocycline treatment. Meanwhile, SB203580, a selective inhibitor of p38, inhibited the mRNA expression of tyrosinase and TRP-1 (Fig. 4).

Phosphorylation of ERK in Melanoma Cells Following Minocyline Treatment To investigate the minocyline-induced phosphorylation of ERK, we performed Western blot analysis of lysate from melanoma cells incubated with 5 μg/ml of minocycline for 5 to 180 min. Minocyline caused an increase in ERK phosphorylation that reached high levels at 30–60 min. However, the increase was transient and little phosphorylation was observed after 90 min of incubation (Fig. 5).

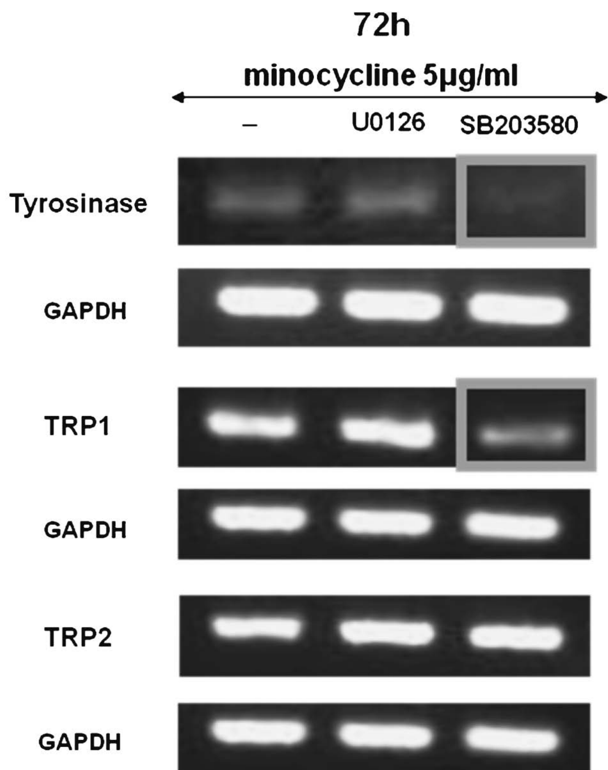

Fig. 4. Effect of U0126 and SB203580 on mRNA Expression of Tyrosinase, TRP-1 and TRP-2

Cells were pre-treated with 10 μM of U0126 or SB203580 for 1 h and then cultured with 0, 1, 5, or 10 μg/ml of minocycline for 72 h. Total RNA isolated from cells was reverse transcribed, and subjected to PCR. The PCR products were electrophoresed on a 1% agarose gel and stained with ethidium bromide.

DISCUSSION

It has been reported that there are several types of minocycline-induced hyperpigmentation.[21] Minocycline itself and/or its iron chlate may induce melanin deposition because iron and minocycline were reported to be detected in the pigmented skin after minocycline therapy.[22] Although the above events were confirmed, the mechanism by which minocycline induces pigmentation in tissues has not been entirely elucidated. In this study, we confirmed that the melanin levels in B16 melanoma cells were increased following minocycline treatment. Before examining the melanin levels, we checked the cytotoxicity of minocycline using the MTT assay. Figure 1 shows that minocycline was toxic to B16 cells dose-dependently at concentrations above 5 μg/ml. The cell viability at over 5 μg/ml was significantly decreased compared to the control. So, the intracellular melanin content should have been examined at concentrations below 5 μg/ml. We confirmed that the melanin content significantly increased at the concentration of 5 μg/ml minocycline compared with the control, but did not significantly increase at concentrations below 2.5 μg/ml (Fig. 2). The tyrosinase gene family has been shown to play an important role in the regulation of melanogenesis.[26,27] Since melanin biosynthesis is catalyzed by tyrosinase, and TRP-1 and TRP-2 which act downstream of tyrosinase, the expression of the

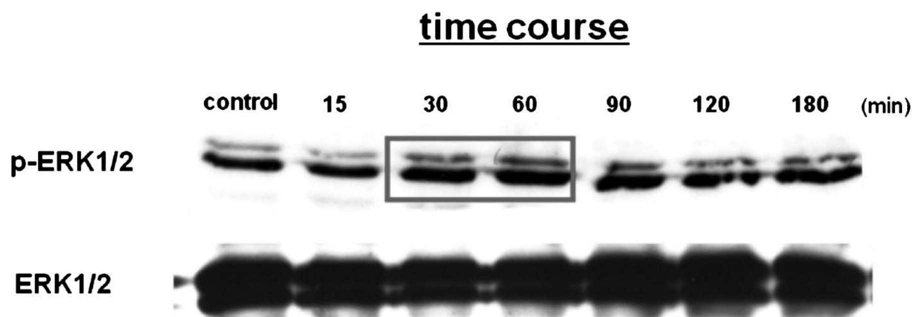

Fig. 5. Effect of Minocycline on ERK1/2 Phosphorylation

B16 melanoma cells were incubated with 5 μg/ml of minocycline from 15 to 180 min mark. Activation of ERK1/2 was analyzed with a specific antibody against the phosphorylated ERK. Equal protein loading was confirmed using the ERK1/2 antibody.

mRNA for each enzyme was examined by RT-PCR. The mRNA levels of tyrosinase, TRP-1, and TRP-2 were increased at 72 h after the treatment with minocyline (Fig. 3). These results indicate that the minocycline-induced melanogenesis in B16 melanoma cells was accompanied by the increase in mRNA. In addition, the increased expression of TRP-1 and TRP-2 mRNA indicated that these two tyrosinase genes also play an important role in minocycline-induced melanogenesis.

MITF is a member of the basic-helix-loop-helix-leucine zipper (bHLH-LZ) family of transcription factors. MITF binds to the M box in the gene promote of tyrosinase and tyrosinase-related proteins and initiates the transcription of genes required for melanin synthesis.[37,38] The proposed scheme of signaling pathways is illustrated in Fig. 6. ERK is one of the participants in an upstream signaling pathway regulating MITF that increases tyrosinase gene expression. There are reports that ERK phosphorylation may induce the degradation of MITF and down-regulate melanogenesis.[39–41] However, there seemed to be little phosphrylation of ERK by minocycline at between 30 and 60 min (Fig. 5). There is a report that ERK activation inhibits melanin synthesis through MITF degradation.[39] Phosphorylated-ERK1/2 down-regulates MITF expression through phosphorylation at serine 73 and subsequent degradation.[42] Another paper also described that phosphorylation of ERK decreased MITF protein levels, and both tyrosinase activity and melanin levels.[43] Our results showed that the ERK phosphorylation was only transient and after that, sustained a control level. We did not examine ERK phosphorylation after the time point of 180 min. However, considering the above reports, little ERK phosphorylation should have occurred after the minocyline treatment in our study. In B16 melanoma cells, the MEK1/2 inhibitor U0126 did not affect the mRNA expression of tyrosinase, TRP-1, or TRP-2 (Fig. 4). The result demonstrated that the slight ERK phosphorylation 30 to 60 min after the minocycline treatment was transient, and did not affect the MITF protein level. However, a p38 inhibitor, SB203580, suppressed expression of the mRNA of tyrosinase and TRP-1 (Fig. 4). MITF is reported to be a direct target of the p38MAPK signaling pathway that is necessary, for example, in osteoblastic differentiation,[33] lipopolysaccharide (LPS)-induced pigmentation in melanocytes,[44] and 2,3,5,4′-tetrahydroxystilben-2-O-beta-D-glucoside (THSG)-induced melanogensis.[45] Signaling *via* p38 has been found to lead to increased phosphorylation and activation of cyclic adenosine

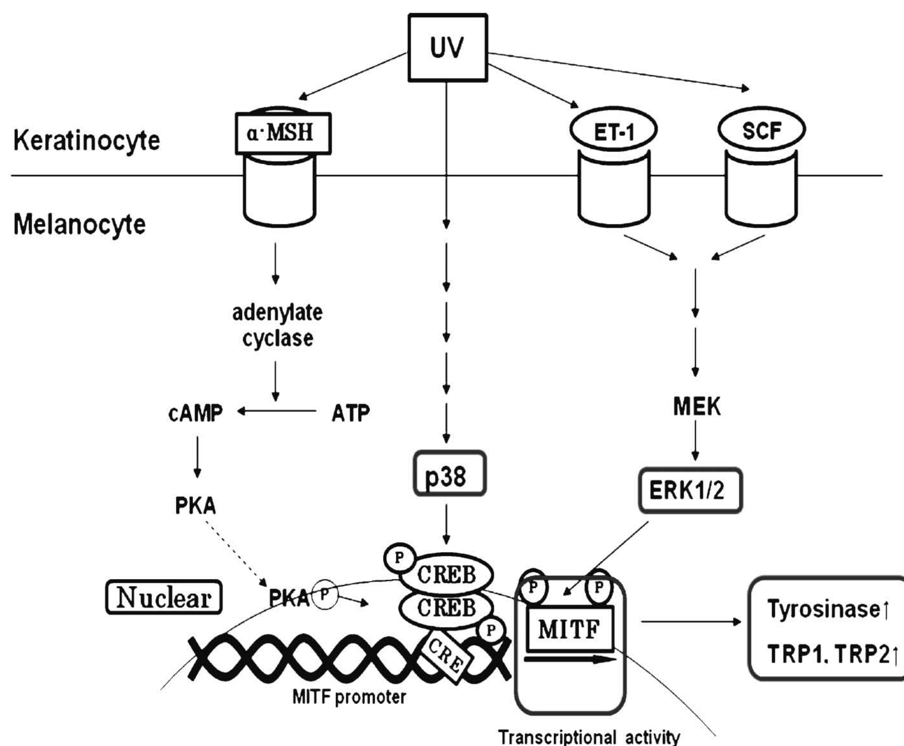

Fig. 6. Scheme of Signaling Pathways Associated with Melanogenesis

monophosphate (cAMP) response element-binding (CREB) that binds and activates the MITF promoter.[46] In this study, although considerable variation was found, we confirmed that the MITF protein level increased after 1 or 5 μg/ml minocycline treatment (data not shown). From the above, the p38 MAPK signaling pathway was suggested to be related to the increase in the MITF protein level.

In conclusion, our findings indicate that minocycline-induced melanogensis occurs *via* an increase in the expression of tyrosinase, TRP-1, and TRP-2, and may involve the activation of p38 MAPK. Further examination concerning p38 MAPK is needed to elucidate minocyline-induced melanogensis. Our results are of help for investigating inhibitors of minocycline-induced pigmentation.

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