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Additive Effects of Heat and p38 MAPK Inhibitor Treatment on Melanin Synthesis

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It has been reported that the activation of extracellular signal-regulated kinase (ERK) reduces melanin synthesis. Recently, we also found that heat treatment induces ERK activation and inhibits melanogenesis in Mel-Ab cells (a mouse melanocyte cell line). In addition, it was reported that p38 MAPK (mitogen-activated protein kinase) inhibition blocks melanogenesis. Thus, we investigated the effects of heat and of the p38 MAPK inhibitor, SB203580, on melanogenesis. In this study, we found that heat treatment activates ERK and reduces melanin production in human melanocytes, and that this is accompanied by a reduction in tyrosinase activity. To regulate the ERK and p38 MAPK pathways simultaneously, we combined heat treatment and SB203580 and measured melanin synthesis. The results obtained showed that heat treatment and SB203580 reduced melanin synthesis more effectively than heat or SB203580 alone. We conclude that ERK activation and p38 MAPK inhibition can work in an additive manner to decrease melanogenesis.

Key words: Heat, Melanogenesis, p38 MAPK, ERK, Tyrosinase

Abbreviations: ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MITF, microphthalmia-associated transcription factor

INTRODUCTION

The production and distribution of melanin pigment is a major determinant of skin and hair color. Increased melanin synthesis is not only a characteristic feature of many hyperpigmentary skin diseases, such as melasma, freckles, and solar lentigo, but also causes many cosmetic problems (Briganti *et al.*, 2003). In efforts to resolve these problems, many have focused on the screening of skin whitening agents. In terms of melanin synthesis, tyrosinase plays a key role because it catalyses the rate-limiting reactions of melanogenesis (Hearing and Jimenez, 1989). Thus, many researchers have searched for potent tyrosinase inhibitors. However, these inhibitors often have side effects, like skin irritation or permanent depigmentation. In addition, some inhibitors need unacceptably high doses to produce visible effects. Therefore, the regulations of melanin synthesis signal transduction pathways are now the focus of attention with respect to treating hyperpigmentation.

Human skin is constantly exposed to heat. Heat is known as an environmental factor to affect skin pigmentation, but its effects on melanogenesis have been poorly studied. Recently, we reported that heat treatment reduced melanin synthesis in a spontaneously immortalized mouse melanocyte cell line Mel-Ab *via* protein phosphatase 2A inactivation and subsequent extracellular signal-regulated kinase (ERK) activation (Kim *et al.*, 2005). Generally, the ERK pathway regulates cell proliferation and differentiation in many types of cells (Cowley *et al.*, 1994; Marshall, 1995; Sale *et al.*, 1995), and this pathway is also involved in the regulation of melanin synthesis (Englaro *et al.*, 1998). In addition, it is known that microphthalmia-associated transcription factor (MITF) is a key transcriptional regulator of melanocyte pigmentation (Hodgkinson *et al.*, 1993; Steingrimsson *et al.*, 1994). Recently, we demonstrated that ERK activation by sphingosine-1-phosphate, ceramide, or sphingosylphosphorylcholine is responsible for reducing

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melanogenesis by downregulating MITF (Kim *et al.*, 2002, 2003, 2006), and thus, we suggested that the ERK pathway could control melanogenesis.

In general, the p38 MAPK (mitogen-activated protein kinase) pathway can be stimulated by various stresses including heat, and it mediates stress-induced signals (Harper and LoGrasso, 2001; Zanke *et al.*, 1996). Furthermore, this pathway is also involved in the melanogenic effects of α-melanocyte stimulating hormone (Smalley and Eisen, 2000), which suggests that p38 MAPK inhibition is a potential target for skin whitening.

Melanogenesis is regulated by the balances of a variety of signal transduction pathways. As mentioned, heat treatment induces the ERK activation, which decreases melanin synthesis. On the other hand, heat also activates p38 MAPK, which can stimulate melanin production. As we found previously, the net effect of heat is decreasing melanin synthesis (Kim *et al.*, 2005). This indicates that the effect of the ERK pathway overwhelms the p38 MAPK activation. Therefore, to maximize the melanin-decreasing effect of heat, we used a specific p38 MAPK inhibitor, SB203580. In the present study, we investigated the additive effects of heat treatment and p38 MAPK inhibition on tyrosinase activity and melanin synthesis. The results obtained indicate that the ERK activation/p38 MAPK inhibition combination could be used to reduce melanin synthesis.

MATERIALS AND METHODS

Materials

SB203580 was obtained from from Calbiochem (San Diego, CA), and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), cholera toxin (CT), synthetic melanin, L-DOPA, and mushroom tyrosinase from Sigma (St. Louis, MO). Antibodies recognizing phospho-specific ERK1/2 (Thr202/Tyr204, number 9101S), total (phosphorylated and non-phosphorylated) ERK1/2 (number 9102), and phospho-specific p38 MAPK (Thr180/Tyr182, # 9211S) were purchased from Cell Signaling Technology (Beverly, MA); and total p38 (A-12, sc-7972) and actin (I-19, sc-1616) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cell cultures and treatment

The Mel-Ab cell line used in this study is a mouse-derived spontaneously immortalized melanocyte cell line, which produces large amounts of melanin (Dooley *et al.*, 1994). Mel-Ab cells were incubated in DMEM supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT), 100 nM TPA, 1 nM CT, 50 μg/mL streptomycin, and 50 μg/mL penicillin at 37°C in 5% CO_2. On the other hand, human epidermal melanocytes were isolated from adolescent foreskins, as previously described (Eisinger and Marko, 1982). Cells were maintained in modified MCDB 153 (Sigma), as previously described (Medrano and Nordlund, 1990), supplemented with 5% FBS, 13 μg/mL bovine pituitary extract (Sigma), 10 ng/mL TPA (Sigma), 5 μg/mL insulin (Sigma), 0.5 μg/mL transferrin (Sigma), 1 mg/mL tocopherol (Sigma), 0.5 μg/mL hydrocortisone (Sigma), 1 ng/mL human recombinant basic fibroblast growth factor (Sigma), and 1% penicillin-streptomycin (10,000 U/mL and 10,000 μg/mL, respectively) (Gibco BRL). Cells were maintained in a humidified incubator in 5% CO_2 at 37°C. Second and third passage melanocytes were used in the experiments. Cells were heat treated by incubating culture dishes in a separate incubator in 5% CO_2 for 1 h at 44°C.

Cell viability assays

Cell viabilities were determined using crystal violet assays (Dooley *et al.*, 1994). Briefly, after heat treatment, cells were incubated in 5% CO_2 for 24 h at 37°C, and then culture media were removed and replaced with 0.1% crystal violet in 10% ethanol for 5 minutes at room temperature. The cells were then rinsed four times in distilled water, and the crystal violet retained by adherent cells was extracted with 95% ethanol. Absorbances were determined at 590 nm using an ELISA reader (TECAN, Salzburg, Austria).

Measurement of melanin contents and microscopy

Melanin contents were measured as described previously (Tsuboi *et al.*, 1998) with slight modification. Briefly, after heat treatment, cells were cultured for 4 days. Cell pellets were then dissolved in 1 mL of 1N NaOH at 100°C for 30 minutes and centrifuged for 20 minutes at 16,000 g. Optical densities (OD) of supernatants were measured at 400 nm using an ELISA reader. Standard curves of synthetic melanin (0 to 300 μg/mL) were prepared for each experiment. Before measuring melanin content, cells were observed under a phase contrast microscope (Olympus Optical Co., Tokyo) and then photographed with a CoolSNAP$_{cf}$ digital video camera system (Roper Scientific, Inc., Tucson, AZ). Image manipulation was conducted using RS Image software (Roper Scientific, Inc., Tucson, AZ).

Tyrosinase activity

Tyrosinase activity was determined as previously described (Busca *et al.*, 1996) with slight modification. Briefly, cells were cultured in 60 mm dishes. After heat treatment, cells were incubated for 4 days, and then washed with ice-cold PBS and lysed with phosphate buffer (pH 6.8) containing 1% Triton X-100. The cells were then disrupted by freezing and thawing, and the lysates obtained were clarified by centrifugation at 10,000 g for 5

minutes. After quantifying protein levels and adjusting protein concentrations with lysis buffer, 90 μL of each lysate, containing the same amounts of protein, were placed in separate wells in a 96-well plate, and 10 μL of 10 mM L-DOPA was then added per well. Control wells contained 90 μL of lysis buffer and 10 μL of 10 mM L-DOPA. The absorbances at 37°C were measured every 10 minutes over at least 1 h at 475 nm using an ELISA reader.

Western blot analysis

Cells were lysed in cell lysis buffer [62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, protease inhibitors (Complete™, Roche, Mannheim, Germany), 1 mM Na_3VO_4, 50 mM NaF, and 10 mM EDTA]. Ten micrograms of protein per lane was separated by SDS-polyacrylamide gel electrophoresis and blotted onto PVDF membranes, which were then saturated with 5% dried milk in Tris-buffered saline containing 0.4% Tween 20. Blots were incubated with the appropriate primary antibodies at a dilution of 1:1000, and then further incubated with horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using enhanced chemiluminescence plus kits (Amersham International, Little Chalfont, U.K.).

Statistics

Differences were assessed for significance using the Student's t-test.

RESULTS

Effects of heat treatment on tyrosinase activity and melanin synthesis in human melanocytes

After heat treatment, human melanocytes were cultured at 37°C for 4 days. Since tyrosinase is the rate-limiting enzyme during melanin synthesis, tyrosinase activity was first determined in heat-treated cells. As shown in Fig. 1A, tyrosinase activity was significantly reduced by heat treatment. We also measured the melanin contents of human melanocytes after heat treatment and found that these were reduced significantly. In addition, crystal violet cell viability assays indicated that heat treatment at the temperatures used (40-44°C) was not cytotoxic to human melanocytes (data not shown).

Heat treatment induced the activation of ERK and p38 MAPK in human melanocytes

ERK phosphorylation is a key feature for reduced melanin synthesis (Hemesath *et al.*, 1998; Wu *et al.*, 2000). Thus, we examined whether heat treatment influences the ERK pathway. As shown in Fig. 2, heat treatment induced ERK activation strongly in human melanocytes. In addition, p38 MAPK was activated slightly.

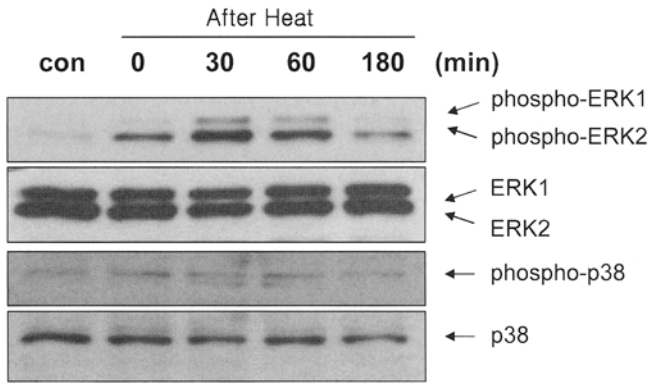

Fig. 2. Heat treatment stimulates ERK and p38 MAPK in human melanocytes. After serum starvation for 24 h, human melanocytes were stimulated at 44°C for 1 h and then transferred to the incubator at 37°C. Whole cell lysates were then Western blotted with antibodies against phospho-specific ERK and phospho-specific p38 MAPK. Equal protein loadings were confirmed using phosphorylation-independent ERK and p38 MAPK antibodies.

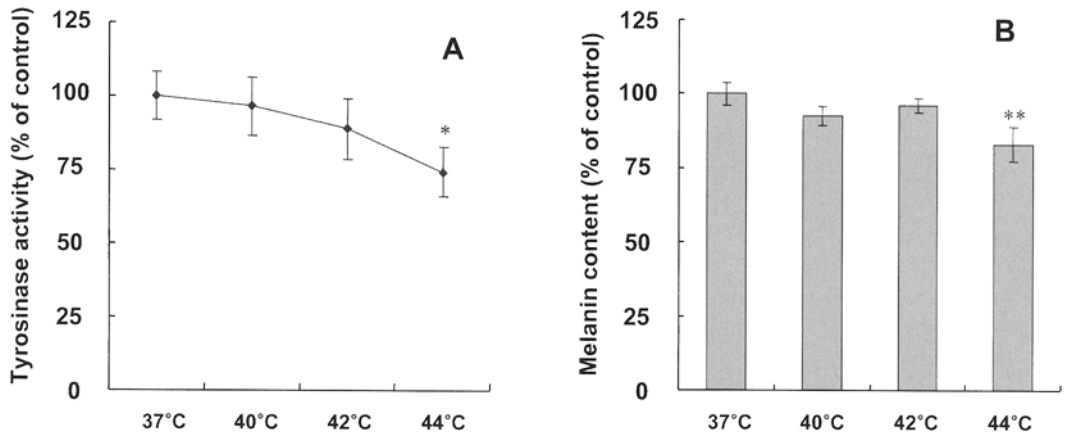

Fig. 1. Effects of heat treatment on melanogenesis in human melanocytes. After heat treatment at the indicated temperatures for 1 h, human melanocytes were cultured at 37°C for 4 days, and tyrosinase activities (A) and melanin contents (B) were measured, as described in "Materials and Methods". The results shown are averages of three independent experiments ± S.D. $^{**}p < 0.01$, $^{*}p < 0.05$ compared to the untreated control.

Effect of heat treatment and/or p38 MAPK inhibition on melanin synthesis

As shown in Fig. 2, heat also activates p38 MAPK, which can stimulate melanin production. Thus, to optimize the melanin-decreasing effect of heat, we hypothesized that a combination of treatment with heat and p38 inhibitor would lead to substantial melanin decrease. Thus, heat-treated Mel-Ab cells were cultured for 4 days in the absence or presence of SB203580 (a specific inhibitor of the p38 MAPK pathway) and then photographed under a phase contrast microscope (Fig. 3A). SB203580-treated cells were found to be less pigmented than control cells, and cells co-treated with SB203580 and heat were much less pigmented than cells treated with SB203580 or heat alone. Melanin synthesis and tyrosinase activity were also measured after SB203580 and/or heat treatment, and consistent with our microscopic observations, SB203580 and heat treatment showed additive inhibitory effects on tyrosinase activity and melanin synthesis (Fig. 3B, C).

To verify the effects of SB203580 on the signaling pathways, we carried out the ERK and p38 MAPK activation experiment using SB203580. In agreement with Fig. 2, we found that heat treatment at 44°C induced ERK and p38 MAPK activation (Fig. 3D, lane 2). SB203580 had no influence on the ERK activation. However, it abrogated heat-induced p38 MAPK activation (Fig. 3D, lane 4). These results suggest that ERK activation and p38 MAPK inhibition both downregulate melanin synthesis co-operatively.

DISCUSSION

Recently, we reported that heat treatment reduced

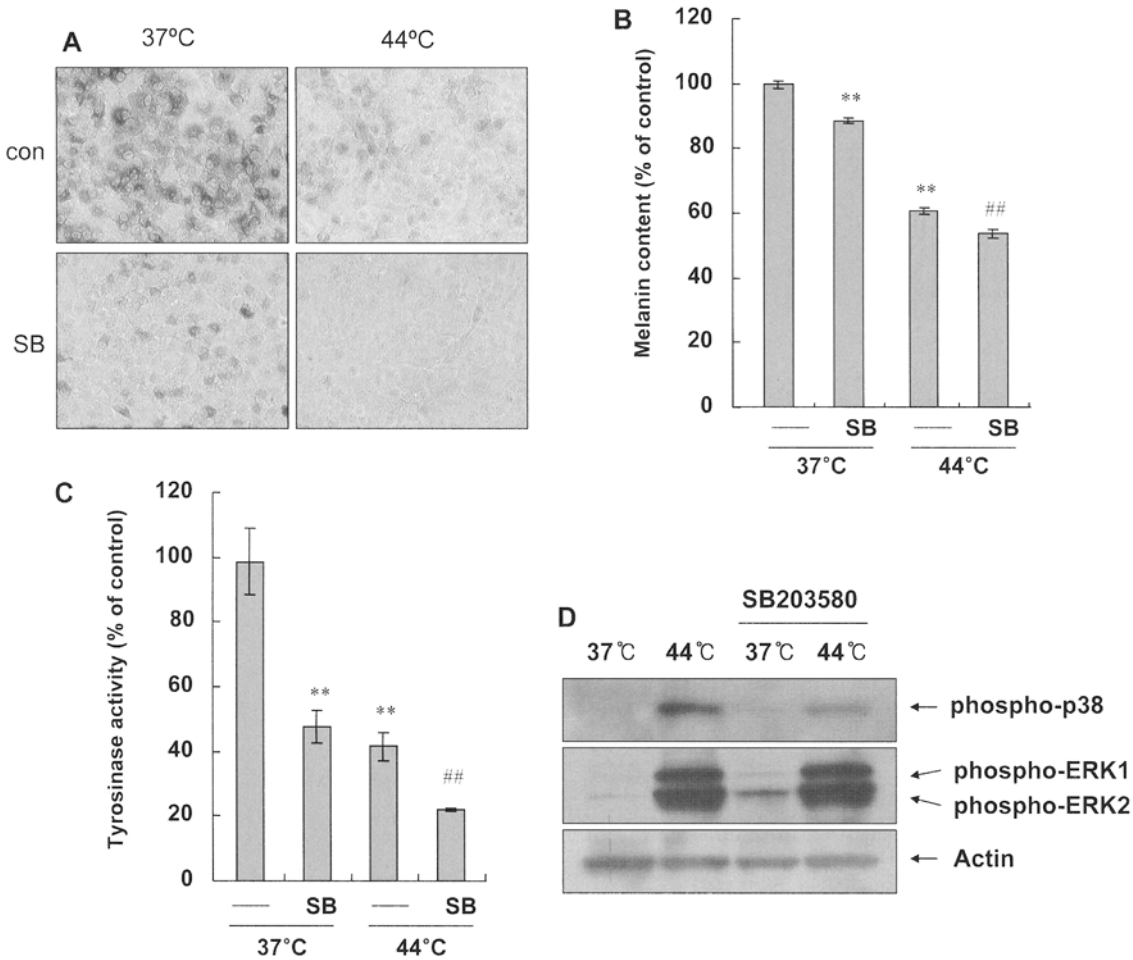

Fig. 3. Effects of heat and/or p38 inhibitor on melanogenesis. In the absence or presence of 5 μM of SB203580, Mel-Ab cells were heat treated (at 44°C for 1 h), and then cultured for 4 days. Phase contrast pictures were taken using a digital video camera (A), and melanin contents (B) and tyrosinase activities (C) were measured as described in "Materials and Methods". Values represent the means of three independent experiments±SD. $^{**}p < 0.01$ compared to the untreated control. $^{\#\#}p < 0.01$ compared to the SB203580-treated group. (D) After serum starvation for 24 h, 5 μM of SB203580 was pretreated for 1 h prior to heat treatment. Mel-Ab cells were then stimulated for 1 h at 44°C. Whole cell lysates were then Western blotted with antibodies against phospho-specific ERK and phospho-specific p38 MAPK. Equal protein loadings were confirmed using anti-actin antibody.

melanin synthesis in Mel-Ab cells (Kim *et al.*, 2005), and in the present study, we found that melanin synthesis is also reduced in human melanocytes by heat. These findings suggest that the effect of heat on melanogenesis is not cell-type specific. However, melanogenesis is a more protracted process in human melanocytes than in Mel-Ab cells, and the hypopigmentary effect of heat was relatively weak in human melanocytes.

Previously, we suggested that heat-induced hypopigmentation is related to ERK activation (Kim *et al.*, 2005). Moreover, it has been reported that ERK activation inhibits tyrosinase gene transcription (Englaro *et al.*, 1998), and that the inhibition of the ERK pathway increases melanin production in human melanoma cells (Koo *et al.*, 2002). We have also reported that PD98059, a specific ERK pathway inhibitor increases melanin production in human and mouse melanocytes (Kim *et al.*, 2002, 2003, 2004; Park *et al.*, 2004). These previous findings indicate that the activation of the ERK pathway can lead to the inhibition of melanogenesis. In the present study, heat treatment was found to induce ERK activation and to reduce melanin synthesis in human melanocytes, thus indicating that heat-induced ERK activation can induce skin whitening.

The inhibition of p38 MAPK with SB203580 was found to block the melanogenic effects induced by α-melanocyte stimulating hormone (Smalley and Eisen, 2000). Furthermore, p38 MAPK is known to be involved in ultraviolet-induced tyrosinase expression (Galibert *et al.*, 2001), and that human placental lipid induces p38 MAPK activation, subsequent tyrosinase expression, and increases melanogenesis (Singh *et al.*, 2005). Thus, p38 MAPK inhibition might present a new strategy for reducing melanin synthesis. In the present study, heat treatment activated p38 MAPK in human melanocytes and Mel-Ab cells, which might have increased melanin synthesis (Fig. 2, 4D), suggesting that p38 MAPK inhibition and heat treatment would achieve an additive hypopigmentary effect. Our results show that co-treatment with SB203580 and heat had an additive inhibitory effect on tyrosinase activity and melanin synthesis.

In summary, the present study demonstrates that heat treatment reduces melanin synthesis *via* ERK activation in human melanocytes. In addition, it was found that combined treatment with a p38 MAPK inhibitor and heat dramatically reduced melanin synthesis. Based on these results, we propose that modalities based on the co-regulation of p38 inhibition and ERK activation offer a powerful means of skin whitening.

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