Arch Pharm Res Vol 26, No 10, 840-845, 2003

Archives of Pharmacal Research
http://apr.psk.or.kr

Temperature Regulates Melanin Synthesis in Melanocytes

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(Revised October 20, 2003)

Temperature change is one of the major environmental factors that influence the human skin. However, the relationship between temperature and melanogenesis has received little attention. In the present study, we investigated the effects of temperature change on melanogenesis in a mouse melanocyte cell line (Mel-Ab), and primary cultured human melanocytes. We found that Mel-Ab cells cultured at low temperatures (31 and 34°C) produce less melanin than cells at 37°C. These results were confirmed by experiments upon human melanocytes, demonstrating that the hypopigmenting effect of low temperatures is not cell type dependent. The observed melanin production was found to be accompanied by tyrosinase activity at each temperature, indicating that tyrosinase activity is regulated by temperature. We further examined whether the incubation period at low temperatures plays an important role in the regulation of melanogenesis. Short exposures to 27°C for 1 h or 3 h did not affect tyrosinase activity or melanin synthesis, whereas long exposures to 31°C for 2 days or 6 days significantly reduced tyrosinase activity and melanin synthesis in a duration-dependent manner. Our results suggest that exposure to low temperature and the duration of this exposure are important regulators of melanogenesis.

Key words: Temperature, Melanogenesis, Tyrosinase, Human melanocytes

INTRODUCTION

Human skin color is determined by melanin pigmentation, which is influenced by environmental factors, like ultraviolet light exposure (Gilchrest *et al.*, 1996; Yanase *et al.*, 2001). Temperature change is a major environmental factor that may influence human skin, and human epidermal keratinocytes are routinely exposed to potential damage by heat, cold or pro-oxidative stress, which result in the induction of cutaneous inflammation (Alappatt *et al.*, 2000). Heat treatment also increases the production of interleukin-1α, prostaglandin E_2, and heat shock protein 70 in artificial human skin (Bowers *et al.*, 1999). However, the relationship between temperature change and melanogenesis has received little attention, though it has been reported that heat treatment enhances melanin synthesis in human melanocytes, thus indicating that heat may regulate melanogenesis in a manner similar to ultraviolet light (Nakazawa *et al.*, 1998).

Temperature of human body is known to be 37°C. However, the temperature of the human skin is substantially lower than body temperature (Mauderli *et al.*, 2003). In this study, we investigated the effects of lower temperatures on melanin synthesis in a spontaneously immortalized mouse melanocyte cell line (Mel-Ab), and in human melanocytes. To our knowledge, this is the first study to investigate the effects of low temperature on melanogenesis. We also measured tyrosinase activity, since tyrosinase is known to catalyze the rate-limiting reactions of melanogenesis (Hearing and Jimenez, 1989). In addition, we monitored mushroom tyrosinase activity changes in a cell-free system to examine its pigmentation regulating effect at different temperatures.

MATERIALS AND METHODS

Materials

MCDB 153, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), cholera toxin (CT), insulin, transferrin, tocopherol, hydro-

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cortisone, synthetic melanin, L-DOPA, and mushroom tyrosinase were purchased from Sigma (St. Louis, MO, USA). Human recombinant basic fibroblast growth factor (bFGF), penicillin-streptomycin, bovine pituitary extract (BPE) were from Gibco BRL (Gaithersburg, MD, USA) and fetal bovine serum (FBS) was from Hyclone (Logan, UT, USA).

Cell cultures and treatment

Mel-Ab is a mouse-derived spontaneously immortalized melanocyte cell line that produces large amounts of melanin (Dooley *et al.*, 1994). Mel-Ab cells were incubated in DMEM supplemented with 10% FBS, 100 nM TPA, 1 nM CT, and penicillin-streptomycin (50 U/mL and 50 μg/mL, respectively) at 37°C in 5% CO_2. For low temperature treatment, another cell culture incubator was used at each temperature.

Human epidermal melanocytes were isolated from adolescent foreskins, as previously described (Eisinger and Marko, 1982). The cells were maintained in modified MCDB 153 as previously described (Medrano and Nordlund, 1990), and supplemented with 5% FBS, 13 μg/mL BPE, 10 ng/mL TPA, 5 μg/mL insulin, 0.5 μg/mL transferrin, 1 μg/mL tocopherol, 0.5 μg/mL hydrocortisone, 1 ng/mL bFGF, and penicillin-streptomycin (50 U/mL and 50 μg/mL, respectively). Cells were maintained in a humidified incubator in 5% CO_2 at 37°C. Second and third passage melanocytes were used in the experiments.

Cell viability assay

Cell viability was determined using a crystal violet assay (Dooley *et al.*, 1994). The culture medium was removed and replaced with 0.1% crystal violet in 10% ethanol for 5 min at room temperature. The cells were then rinsed four times in distillated water, and the crystal violet retained by adherent cells was extracted with 95% ethanol. Absorbance was 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. Cell pellets were dissolved in 1 mL of 1 N NaOH at 100°C for 30 min and centrifuged for 20 min at 16,000 g. Optical densities (OD) of the supernatants were measured at 400 nm using an ELISA reader. Standard curves of synthetic melanin (0 to 300 μg/mL) were prepared in triplicate for each experiment. Before measuring the melanin content, the cells were observed under a phase contrast microscope (Olympus Optical Co., Tokyo, Japan) and photographed with a CoolSNAP$_{cf}$ digital video camera system (Roper Scientific, Inc., Tucson, AZ, USA) supported by RS Image software (Roper Scientific, Inc., Tucson, AZ, USA).

Tyrosinase activity

Tyrosinase activity was determined as previously described (Busca *et al.*, 1996), with slight modification. Briefly, Mel-Ab cells were cultured in 60 mm dishes, washed with ice-cold PBS and lysed with phosphate buffer (pH 6.8) containing 1% Triton X-100. Cells were then disrupted by freezing and thawing, and the lysates were clarified by centrifugation at 10,000 g for 5 min. After quantifying the protein levels and adjusting concentrations using lysis buffer, 90 μL of each lysate, containing the same amount of protein, was placed in each well of a 96-well plate, and 10 μL of 10 mM L-DOPA was added to each well. Control wells contained 90 μL of lysis buffer and 10 μL of 10 mM L-DOPA. Following incubation at 37°C, absorbance at 475 nm was measured every 10 min for at least 1 h using an ELISA reader.

A cell-free assay system was used to test the direct effect of temperature on tyrosinase activity. Seventy microliters of phosphate buffer were mixed either with 20 μL of 100 μg/mL mushroom tyrosinase or with 20 μL of human tyrosinase as 20 μg of total protein extracted from primary cultured human melanocytes, and 10 μL of 10 mM L-DOPA. Following incubation at temperatures ranging from 27°C to 37°C, absorbance was measured at 475 nm.

Statistics

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

RESULTS

Effects of temperature on tyrosinase activity and melanin synthesis in Mel-Ab cells

We used two identical CO_2 incubators to culture Mel-Ab cells at different temperatures. In one incubator, cells were cultured at 37°C for 4 days as a control. Two other groups of cells were cultured at 34°C for 2 days in the other incubator. Thereafter, one group was returned to 37°C incubator, whereas the other group was kept in the other incubator that was set to 31°C. Both groups of cells were then cultured for a further 2 days.

Phase contrast microphotographs showed that melanin pigmentation was reduced at the lower temperatures, i.e., 34 and 31°C (Fig. 1A). The melanin contents of the Mel-Ab cells were also measured. As shown in Fig. 1B, melanin contents were reduced significantly at the lower temperatures (34 and 31°C). Moreover, this melanin down-regulation at low temperatures followed a temperature dependent reduction in tyrosinase activity (Fig. 1C). These results indicate that temperature regulates tyrosinase, and that this is followed by the inhibition of melanin synthesis in Mel-Ab cells.

We further confirmed these low temperature-induced

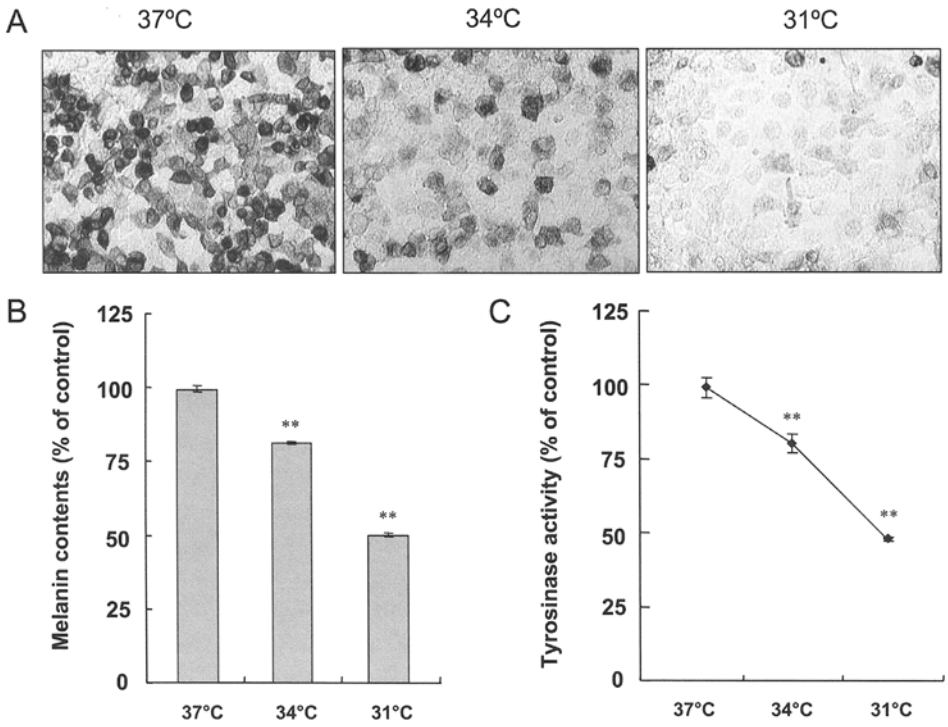

Fig. 1. Effects of low temperatures on the melanogenesis of Mel-Ab cells. Cells were cultured at the indicated temperatures for 4 days (refer to the text for details). (A) Phase contrast pictures were taken using a digital video camera. Tyrosinase activity (B) and melanin contents (C) were measured, as described in "Materials and Methods". Results are the averages of three independent experiments±SD. **P<0.01 compared to control.

effects in primary cultured human melanocytes. Under our culture conditions, human melanocytes exhibited a bipolar morphology. However, low temperature treatment induced a morphologic change; the cells became larger bodied (Fig. 2A). We next measured the melanin contents of human melanocytes cultured at different temperatures. Similar results were obtained for human melanocytes as had been obtained with Mel-Ab cells; for example, incubation at low temperature was found to inhibit melanin synthesis (Fig. 2B). These results indicate that the low temperature-

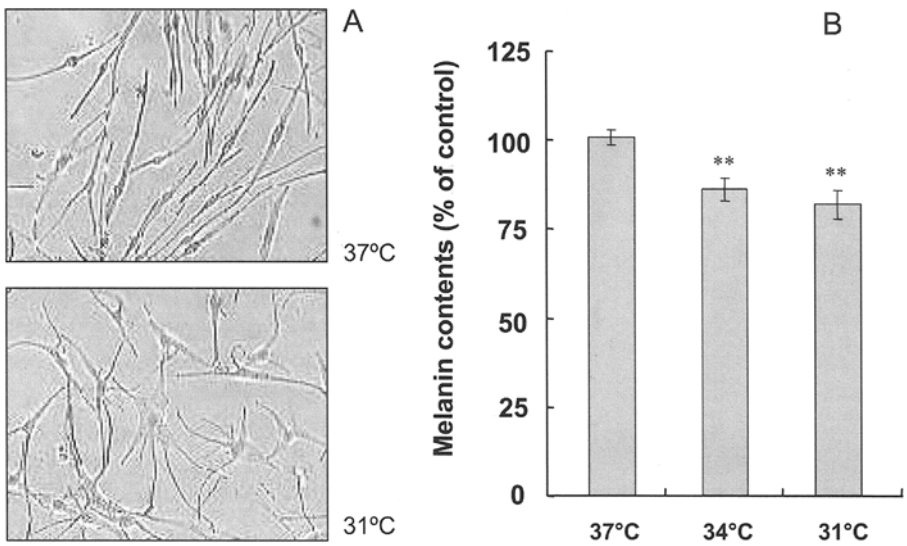

Fig. 2. Effects of low temperatures on the melanin synthesis of human melanocytes. Cells were cultured at the indicated temperatures for 4 days (refer to the text for details). (A) Phase contrast pictures were taken using a digital video camera. Melanin contents (B) were determined, as described in "Materials and Methods". Results are the averages of three independent experiments±SD. **P<0.01 compared to control.

mediated hypopigmentation is not specific for Mel-Ab cells.

To examine whether a low temperature leads to cell death, we incubated cells at 37-31°C, and determined cell viability after incubating for 24 h using the crystal violet assay. No cell viability differences were observed over the range 37 to 31°C (data not shown).

Effects of the duration of exposure to low temperature on tyrosinase activity and melanin synthesis in Mel-Ab cells

In one incubator, Mel-Ab cells were cultured at 37°C for 6 days as a control group. In order to test the effects of short-term treatment to low temperature, two groups of Mel-Ab cells were incubated at 27°C for 1 h and 3 h, respectively, and then returned to a 37°C incubator and maintained for 6 days. To test the effects of long-term treatment to low temperature, Mel-Ab cells were cultured at 31°C for 2 days, and then split into two groups. One group was cultured in a 37°C incubator for 4 days, whereas the other was cultured at 31°C for the same period. The cells were then photographed under a phase contrast microscope. The photomicrographs obtained showed that the melanin pigmentation was reduced in a duration-dependent manner at the lower temperatures (Fig. 3A). In line with these results, we also found that a significant reduction in tyrosinase activity occurred by incubating for 2 days or 6 days at 31°C but not for 1 h or 3 h at 27°C (Fig. 3B). We also measured the melanin contents of the Mel-Ab cells, and as is shown by Fig. 3C, melanin reduction due to exposure to 31°C for 2 days or 6 days followed a decrease in the tyrosinase activity. These results indicate that incubation at low temperature regulates tyrosinase, which subsequently inhibits melanin synthesis in Mel-Ab cells.

Effects of temperature on tyrosinase activity in a cell-free system

To explain the effects of low temperatures on melanin synthesis, we investigated the activity of tyrosinase in a

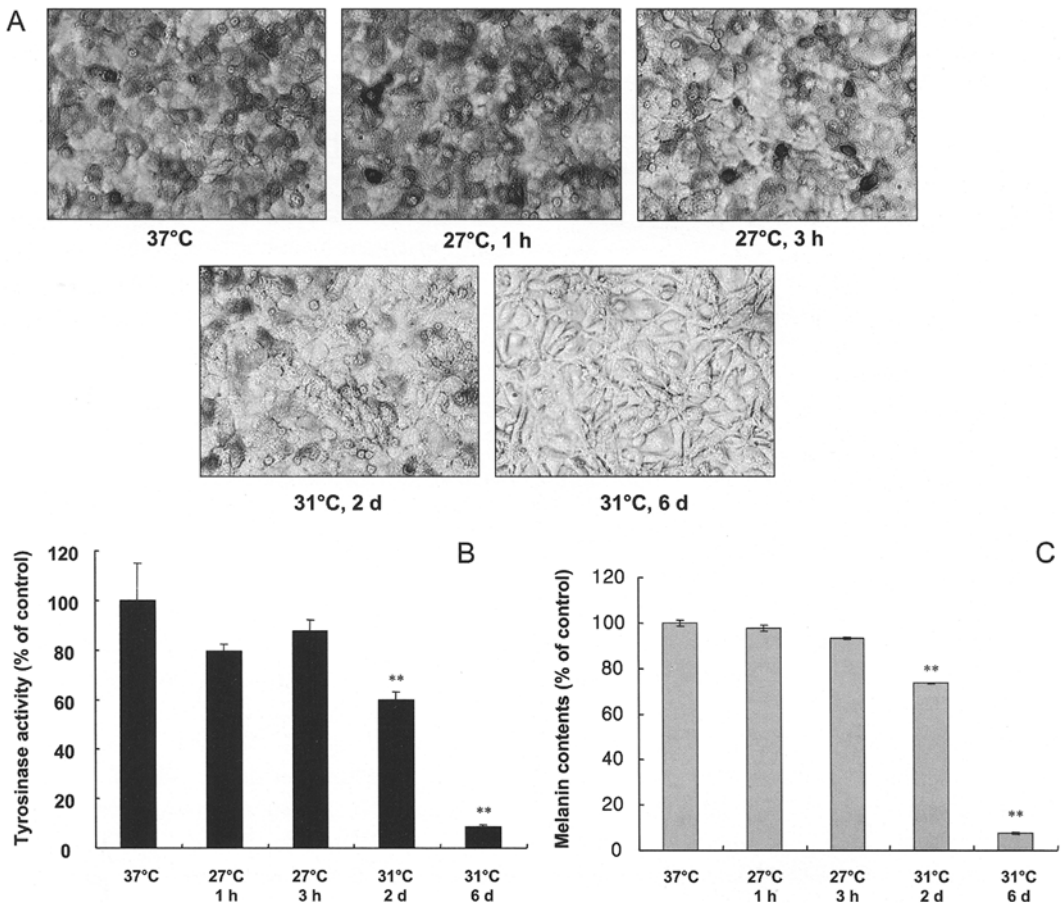

Fig. 3. Effects of the duration of low temperature exposure on melanogenesis. The cells were cultured at the indicated temperatures and times (refer to the text for details). (A) Phase contrast pictures were taken using a digital video camera. Tyrosinase activity (B) and melanin contents (C) were measured, as described in "Materials and Methods". The results shown represent the averages of three independent experiments±SD. **P<0.01 versus the control.

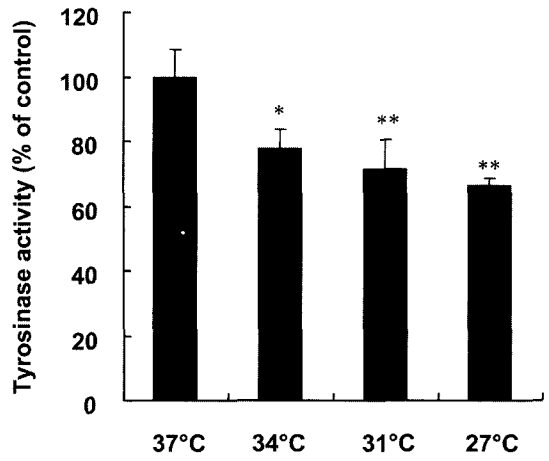

Fig. 4. Effects of temperature on mushroom tyrosinase activity *in vitro*. Mushroom tyrosinase activity was measured in a cell-free system at different temperatures, as described in "Materials and Methods". The results shown are the averages of three independent experiments±SD. *P<0.05, **P<0.01 compared to the control.

cell-free system with respect to temperatures. As shown in Fig. 4, mushroom tyrosinase activity was found to reduce in a temperature-dependent manner, indicating that tyrosinase activity is temperature dependent. We also performed the same experiment on a human melanocyte extract containing human tyrosinase, and obtained similar results (data not shown).

DISCUSSION

Temperature change is an important extracellular stress, but the effects of temperature on melanin synthesis have been little studied. Recently, it was reported that heat treatment increases tyrosinase activity slightly in human melanocytes (Nakazawa *et al.*, 1998). Since skin temperature (about 31°C) is lower than body temperature (Mauderli *et al.*, 2003), we investigated whether low temperature could influence melanin synthesis. Our results show that melanin synthesis is reduced in a temperature-dependent manner, and that tyrosinase activity correlates with melanin content at each temperature. Therefore, reduced tyrosinase activity may be responsible for the lower pigment content in low temperature-treated cells. Furthermore, we found that the duration of cellular exposure to low temperature is also important in the regulation of melanin synthesis. Moreover, this low temperature-induced reduction in melanin pigment was marked enough to be evident under the phase contrast microscope. Thus, we further studied the possible mechanisms associated with this low temperature-induced hypopigmentation.

It has been reported that the extracellular signal-regulated protein kinase (ERK) and the p38 mitogen-activated protein kinase (MAPK) signaling pathways are deeply involved in the regulation of melanogenesis (Englaro *et al.*, 1998; Kim *et al.*, 2003; Kim *et al.*, 2002; Smalley and Eisen, 2000). Thus, we hypothesized that either the activation of the ERK pathway or the inhibition of p38 MAPK could be responsible for observed melanin reductions. However, we were unable to find any significant changes in ERK or p38 MAPK activation in cells cultured at 37°C or 34°C (data not shown).

We next determined mushroom tyrosinase and human tyrosinase activities in a cell-free system at different temperatures, and found that these enzyme activities were significantly dependent on temperature, which may explain the observed reduction in melanin synthesis at the lower temperatures. MITF is one of the major transcriptional factors of tyrosinase and other melanogenic enzymes (Bentley *et al.*, 1994; Busca and Ballotti, 2000; Tachibana, 2000), and Western blotting showed that MITF protein levels were reduced in cells cultured at 34°C (our unpublished data). However, further study is needed to investigate the relevance of temperature-regulated MITF expression.

In summary, the present study demonstrates that low temperature reduces melanin synthesis in Mel-Ab cells and human melanocytes. Moreover, we found that the duration of exposure to low temperature is also critically important in terms of this regulation of melanin synthesis.

ACKNOWLEDGEMENTS

This study was supported by a grant (No. 04-2002-107) from the Seoul National University Hospital, R.O.K, and by financial support from the Pacific Corporation. We also thank Ms. Jai-Eun Lee and Ms. Sook-Young Kim for their excellent technical assistance.

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