Sphingosine-1-phosphate decreases melanin synthesis via sustained ERK activation and subsequent MITF degradation

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Summary

Sphingosine-1-phosphate (S1P) has emerged as a bioactive lipid modulator that mediates a variety of cell functions. However, the effects of S1P on melanogenesis are not well known. Therefore, we investigated the actions of S1P on melanin synthesis using a spontaneously immortalized mouse melanocyte cell line, Mel-Ab. This study shows that S1P significantly inhibits melanin synthesis in a concentration-dependent manner, and also that the activity of tyrosinase was reduced in S1P-treated cells. In contrast, a specific extracellular signal-regulated protein kinase (ERK) pathway inhibitor, PD98059, increased tyrosinase activity and melanin production, and PD98059 also restored the S1P-induced reduction of tyrosinase activity and pigmentation. In addition, we found that S1P induces the sustained activation of ERK and the subsequent degradation of microphthalmia-associated transcription factor (MITF), which plays a key role in melanogenesis. Thus, we further studied the relationship between the ERK pathway and melanin synthesis. PD98059 was found to prevent the S1P-induced MITF phosphorylation and degradation and to abrogate the S1P-induced downregulation of tyrosinase and of tyrosinase-related protein 1 (TRP1) production. These results indicate that the ERK pathway is potently involved in the melanogenic signaling cascade, and that S1P-induced ERK activation contributes to reduced melanin synthesis via MITF degradation. Therefore, we suggest that S1P reduces melanin synthesis by ERK activation, MITF phosphorylation and degradation, and by the subsequent downregulation of tyrosinase and TRP-1 production.

Key words: Sphingosine-1-phosphate, Melanogenesis, Microphthamia, ERK, MITF

Introduction

Sphingosine-1-phosphate (S1P) is generally believed to participate in the regulation of cell growth, differentiation, migration and programmed cell death (Pyne and Pyne, 2000; Spiegel, 1999). Despite the important roles of S1P in many biological processes, little attention has been paid to the action of S1P on melanogenesis. Recently, we reported that a sphingolipid metabolite, ceramide, reduces melanin synthesis via extracellular signal-regulated protein kinase (ERK) activation in human melanocytes (Kim et al., 2002). The findings of this study suggest that sphingolipid metabolites are also involved in the regulation of melanogenic processes.

Melanogenesis is regulated by at least three melanocyte-specific enzymes, tyrosinase, tyrosinase-related protein 1 (TRP1) and tyrosinase-related protein 2 (TRP2) (Kobayashi et al., 1994; Prota, 1988; Yokoyama et al., 1994). Among these enzymes, tyrosinase is the rate-limiting enzyme and catalyses the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and the oxidation of DOPA to dopaquinone (Hearing and Jimenez, 1989). Thus, the upregulation of tyrosinase was proposed to be responsible for increased melanin production (Hearing and Tsukamoto, 1991). For these reasons, we started to study the effects of the sphingolipid metabolite S1P, on melanin synthesis and tyrosinase activity.

Microphthalmia-associated transcription factor (MITF) is a transcription factor having an essential basic helix-loop-helix-leucine zipper structure, and is believed to regulate melanocyte pigmentation, proliferation, and survival (Hodgkinson et al., 1993; Steingrimsson et al., 1994). Actually, mutations of the MITF gene in humans are known to cause abnormal pigmentation of the skin and hair, as observed in Waardenburg Syndrome type IIA (Hughes et al., 1994; Tachibana, 1997; Tassabehji et al., 1994). In addition, it has been reported that MITF is a major transcriptional regulator of the melanogenic enzymes, tyrosinase, TRP-1 and TRP-2 (Bentley et al., 1994; Bertolotto et al., 1998b; Yasumoto et al., 1997; Yavuzer et al., 1995). Furthermore, cAMP elevating agents such as α-melanocyte-stimulating hormone (α-MSH), forskolin or isobutylmethylxanthine stimulate melanin synthesis (Englaro et al., 1995; Hunt et al., 1994; Jimenez et al., 1988). Also, it is well known that α-MSH potently induces the expression of MITF (Bertolotto et al., 1998a; Bertolotto et al., 1998b; Price et al., 1998). Thus, we investigated the effects of S1P on MITF regulation.

The ERK pathway is a major signaling cascade and plays a crucial role in cell growth control (Marshall, 1995). Interestingly, ERK is also known to be involved in the regulation of cell differentiation, although this depends on cell type (Cowley et al., 1994; Sale et al., 1995). Recently, it was reported that the inhibition of the ERK pathway induces B16 melanoma cell differentiation and increases tyrosinase activity, suggesting that the ERK signaling pathway regulates melanogenesis (Englaro et al., 1998). Furthermore, it was shown that the activation of ERK by c-Kit stimulation phosphorylates MITF at serine 73 (Hemesath et al., 1998), and that the phosphorylation of MITF at serine 73 is followed by MITF ubiquitination and degradation (Xu et al., 2000). Several lines of evidence suggest that S1P regulates the ERK signaling pathway (Cuvillier et al., 1996; Van Brocklyn et al., 2000). Therefore, we hypothesized that S1P may control melanogenesis via the ERK pathway. In this study, we investigated the effects of S1P on melanogenesis in Mel-Ab cells. In particular, we analyzed changes in the ERK signaling pathway and the accompanying MITF regulation.

Materials and Methods

Materials

S1P, sphingosine and N,N-dimethylsphingosine (DMS) were obtained from Alexis (San Diego, CA); PD98059 was from Cell Signaling Technology (Beverly, MA); and fatty-acid-free bovine serum albumin (BSA), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), cholera toxin (CT), kojic acid, synthetic melanin, L-DOPA and mushroom tyrosinase were from Sigma (St Louis, MO). Sphingolipids were added to cells as a complex with 0.4% BSA. Antibodies that recognize phospho-specific ERK1/2 (Thr202/Tyr204, number 9101S), total (phosphorylated and non-phosphorylated) ERK1/2 (number 9102), phospho-specific MEK (Ser217/221, number 9121) and total MEK (number 9122) were purchased from Cell Signaling Technology; microphthalmia Ab-1 (C5, MS-771-P0) and tyrosinase Ab-1 (MS-800-P0) were from NeoMarkers (Fremont, CA); and TRP-1 (G-17) and actin (I-19) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell cultures

Mel-Ab cell line 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% fetal bovine serum (FBS), 100 nM TPA, 1 nM CT, 50 μg/ml streptomycin and 50 μg/ml penicillin at 37°C in 5% CO_2.

Cell viability assay

Cell viability was determined using a crystal violet assay (Dooley et al., 1994). After incubating with the test substances for 24 hours, the culture medium was removed and replaced with 0.1% crystal violet in 10% ethanol. Cells were then stained for 5 minutes at room temperature and rinsed four times. The crystal violet retained by adherent cells was then extracted with 95% ethanol. Absorbance was determined at 590 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA).

Measurement of melanin contents and microscopy

Melanin contents were measured as described previously (Tsuboi et al., 1998) with slight modification. Briefly, cells were treated with the test substances in DMEM containing 2% FBS for 5 days. Cell pellets were dissolved in 1 ml of 1 N NaOH at 100°C for 30 minutes and centrifuged for 20 minutes 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 melanin content was measured, the cells were observed under a phase contrast microscope (Olympus Optical, Tokyo, Japan) and photographed with a digital color video camera TK-C1380 (JVC, Yokohama, Japan) supported by Image-Pro® Plus software (Media Cybernetics, Silver Spring, MD).

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. After incubating with test substances in DMEM containing 2% FBS for 5 days, the cells were 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 minutes. After quantifying protein levels and adjusting concentrations with lysis buffer, 90 μl of each lysate, containing the same amount of protein, was placed in a 96-well plate, and 10 μl of 10 mM L-DOPA was then 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 was measured every 10 minutes for at least 1 hour at 475 nm using an ELISA reader. A cell-free assay system was used to test for direct effects on tyrosinase activity. Seventy microliters of phosphate buffer containing various concentrations of test substances were mixed with 20 μl of human tyrosinase extracted from primary cultured human melanocytes, as 20 μg of total protein and 10 μl of 10 mM L-DOPA. Following incubation at 37°C, absorbance was measured at 475 nm. As a positive control, 80 μl phosphate buffer were mixed with 10 μl of 10 μg/ml mushroom tyrosinase and 10 μl of 10 mM L-DOPA.

Immunoprecipitation assay

Cells were grown in 100 mm culture dishes, starved of serum for 48 hours, treated with S1P as indicated, lysed on ice for 10 minutes in lysis buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 20,000 ***g*** for 10 minutes at 4°C in a microcentrifuge. The lysates were immunoprecipitated using an antibody against ERK1/2 and protein A agarose beads, which were washed three times with cell lysis buffer to eliminate nonspecific binding. The level of MITF protein was measured by immunoblotting.

Western blot analysis

Cells were grown in 100 mm culture dishes, starved of serum for 48 hours, and treated with test substances as indicated. They were then 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 nitrocellulose membranes, which were 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 an enhanced chemiluminescence plus kit (Amersham International, Little Chalfont, UK).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the cells using an RNeasy Mini Kit

(Qiagen, Valencia, CA). Then, 1 μg of RNA was reverse transcribed using the ImProm II Reverse Transcription System (Promega, Madison, WI). The cDNA obtained was amplified with primers to the mouse MITF gene exons 5-8 (5′ exon 5 CCCGTCTCTGGAAA-CTTGATCG and 3′ exon 8 CTGTACTCTGAGCAGCAGGTG). The PCR conditions were 30 cycles of: 94°C for 1 minute, 57°C for 1 minute and 72°C for 2 minutes (Weilbaecher et al., 1998), and the resulting 414 bp PCR products were visualized by electrophoretic separation on 1.5% agarose gels and ethidium bromide staining. Specific primers for actin were added as a control.

Statistics

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

Results

Effects of S1P on tyrosinase activity and melanin synthesis in Mel-Ab cells

Mel-Ab cells were exposed to sphingolipids at various concentrations ranging from 1-10 μM for 24 hours, and cell viability was determined by using the crystal violet assay. S1P did not show any effect on cell viability at the concentrations used, indicating that S1P was not cytotoxic in Mel-Ab cells, while sphingosine and DMS were cytotoxic (Fig. 1).

Tyrosinase is a rate-limiting enzyme in melanin synthesis. To investigate the effect of S1P on pigmentation, we determined the tyrosinase activity, and found that a significant decrease in tyrosinase activity was induced by S1P at concentrations higher than 1 μM (Fig. 2A). The melanin contents of the Mel-Ab cells were also measured. In untreated cells, a constitutive level of pigment was readily detected (20-30 pg/cell). As shown in Fig. 2B, the melanin contents of cells decreased significantly in the S1P range 1-10 μM. Moreover, melanin reduction by S1P was accompanied by a parallel decrease in tyrosinase activity. These results indicate that S1P regulates tyrosinase, and subsequently inhibits melanin

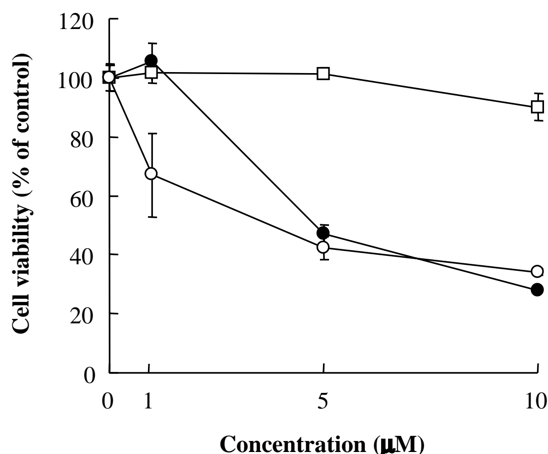

Fig. 1. Cell viability after treating with sphingosine-1-phosphate (□), sphingosine (●) or N,N-dimethylsphingosine (○). After serum starvation, cells were incubated for 24 hours in serum-free media with various concentrations (1-10 μM) of sphingolipids. The viability of the cells was determined by the crystal violet assay. Each determination was made in triplicate and the data represent means ± s.d.

synthesis in Mel-Ab cells. To allow comparisons of the hypopigmenting effects of S1P, kojic acid, which is well known to affect melanin formation in melanocytes and melanoma cells, was added at concentrations from 1 to 100 μM. As expected, kojic acid reduced the amount of melanin production (Fig. 2C). Interestingly, the inhibitory effect of S1P (1-10 μM) on melanin synthesis was greater than that of kojic acid at concentrations in the 1-100 μM range.

To determine whether S1P can inhibit tyrosinase directly, we compared the effect of S1P with that of kojic acid on tyrosinase in a cell-free system, as described above in Materials and Methods, by using human tyrosinase extracted from primary cultured human melanocytes. As shown in Fig. 2D, kojic acid inhibited tyrosinase activity significantly in the cell-free system. In contrast, S1P did not suppress human tyrosinase, indicating that S1P does not inhibit tyrosinase activity directly. We repeated the experiment with mushroom tyrosinase (monophenol monooxygenase, EC 1.14.18.1) and obtained the same result (data not shown).

S1P induces MITF degradation and stimulates the ERK pathway

Because S1P reduced tyrosinase activity and melanin synthesis, we further hypothesized that S1P may affect the expression of MITF, which plays an important role in melanogenesis. To prove this hypothesis, we studied MITF levels after S1P treatment. MITF appeared as a doublet before treatment. S1P treatment induced an initial MITF mobility shift at 2 minutes, and the shift maintained at least until 10 minutes (Fig. 3A). Following this shift, MITF protein levels decreased slowly over the course of several hours.

Previous studies have demonstrated that ERK phosphorylation by steel factor triggered MITF degradation (Hemesath et al., 1998; Wu et al., 2000). Therefore, it was of some interest to determine whether S1P could influence the ERK pathway. As shown in Fig. 3A, S1P induced the sustained activation of ERK. Moreover, the mobility shift of MITF corresponded to strong ERK phosphorylation. We also investigated events upstream of ERK and found that S1P stimulated the phosphorylation of MEK (MAPK/ERK kinase). The kinetics of MEK and ERK activation after S1P stimulation showed similar patterns (Fig. 3A).

Since a decreased MITF gene expression may be responsible for a diminished level of MITF protein, we examined whether S1P has an effect on MITF transcription. Reverse transcription PCR assays using MITF-specific primers produced a 414 bp fragment corresponding to the MITF mRNA. However, we did not observe a lower level of this PCR fragment in S1P-treated cells (Fig. 3B), while the levels of MITF protein dropped significantly (Fig. 3A). Thus, we suggest that the MITF protein reduction by S1P may be due to MITF degradation, not to suppressed MITF gene expression.

Inhibition of the ERK pathway by PD98059 abrogates S1P-induced hypopigmentation

In a recent report, we showed that the sustained activation of ERK could lead to the inhibition of melanin synthesis in human melanocytes (Kim et al., 2002). Therefore, we further studied whether the ERK signaling pathway is related to the regulation

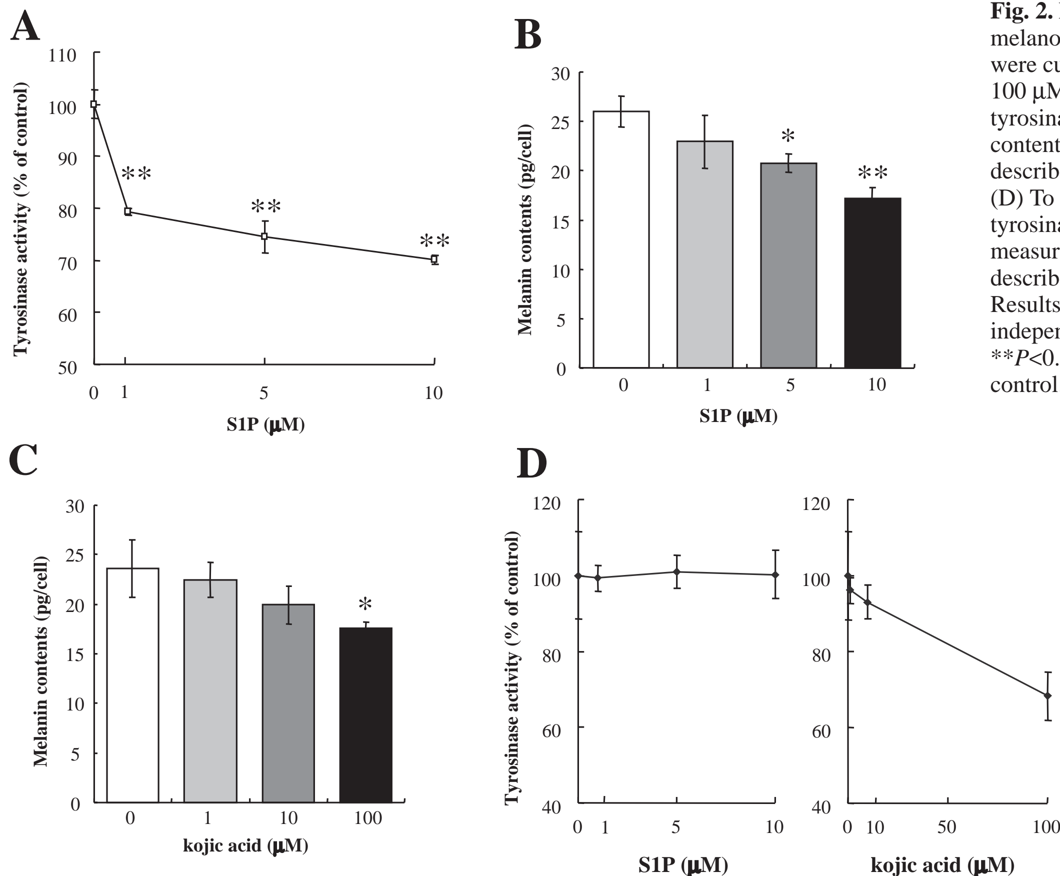

Fig. 2. Effects of S1P and kojic acid on melanogenesis in Mel-Ab cells. Cells were cultured with 1-10 μM S1P or 1-100 μM kojic acid for 5 days, and the tyrosinase activity (A) or melanin content (B,C) was measured, as described in Materials and Methods. (D) To test the direct effect on tyrosinase, tyrosinase activity was measured in a cell-free system, also as described in Materials and Methods. Results are the average of three independent experiments ± s.d. ***P*<0.01, **P*<0.05 compared with control.

of pigmentation. Thus, we investigated the effect of PD98059, a specific inhibitor of the ERK pathway, on melanogenesis. Control and PD98059-treated cells were exposed to S1P for 5 days, and cells were photographed under a phase contrast microscope (Fig. 4A). The melanin pigment was found mainly in the cytoplasm surrounding the nucleus. As we expected from the observed reductions in tyrosinase activity and melanin synthesis after S1P treatment, the S1P-treated cells were much less pigmented than the control cells. In contrast, we observed highly pigmented Mel-Ab cells after PD98059 treatment. Furthermore, PD98059 restored the S1P-induced hypopigmentation to normal pigmentation. We also measured tyrosinase activity and melanin synthesis after treatment with PD98059. Consistent with the microscopic inspection, PD98059 treatment resulted in a significant stimulation of tyrosinase activity and melanin synthesis in Mel-Ab cells, suggesting that the inhibition of ERK may stimulate tyrosinase activity and melanin synthesis (Fig. 4B,C). Conversely, S1P seemed to inhibit tyrosinase activity and melanin synthesis by activating ERK, since S1P led to the sustained activation of ERK (Fig. 3A). Our results also show that the PD98059 pretreatment abolished the inhibitory effect of S1P on tyrosinase activity and melanin synthesis (Fig. 4B,C). These results suggest that the ERK pathway plays a critical role in melanogenesis.

The relationship between the ERK pathway and MITF in Mel-Ab cells

We examined whether ERK phosphorylation by S1P would induce MITF degradation, and thus investigated the nature of the interaction between ERK and MITF by immunoprecipitation. As shown in Fig. 5A, MITF was detected in the ERK immunoprecipitated complex from S1P-treated cells. Moreover, a marked level of mobility-shifted MITF was observed 10 minutes after S1P treatment when ERK was strongly activated (Fig. 5A). This finding shows that S1P induces the formation of a complex between ERK and MITF, and suggests that phosphorylated ERK may be responsible for MITF phosphorylation and degradation.

In the next experiment, Mel-Ab cells were pretreated with 20 μM of PD98059 to inhibit ERK phosphorylation. PD98059 was found to markedly inhibit the S1P-induced activation of ERK (Fig. 5B), and the S1P-dependent mobility shift of MITF, which was expected to appear 10 minutes after S1P treatment (Fig. 5B). Furthermore, we also found that PD98059 abrogated S1P-induced MITF degradation, and that it restored the tyrosinase and TRP-1 downregulation by S1P at 180 minutes (Fig. 5C). These results indicate that a reduction in MITF level is correlated with reduced tyrosinase and TRP-1 levels, and that these responses can be blocked by PD98059 treatment. Our results show that S1P inhibits melanin synthesis via ERK activation. In addition, S1P-induced MITF phosphorylation and degradation are considered to be mediated by the MEK/ERK signaling pathway. From these results, we can conclude that S1P inhibits melanin synthesis through ERK activation and subsequent MITF degradation.

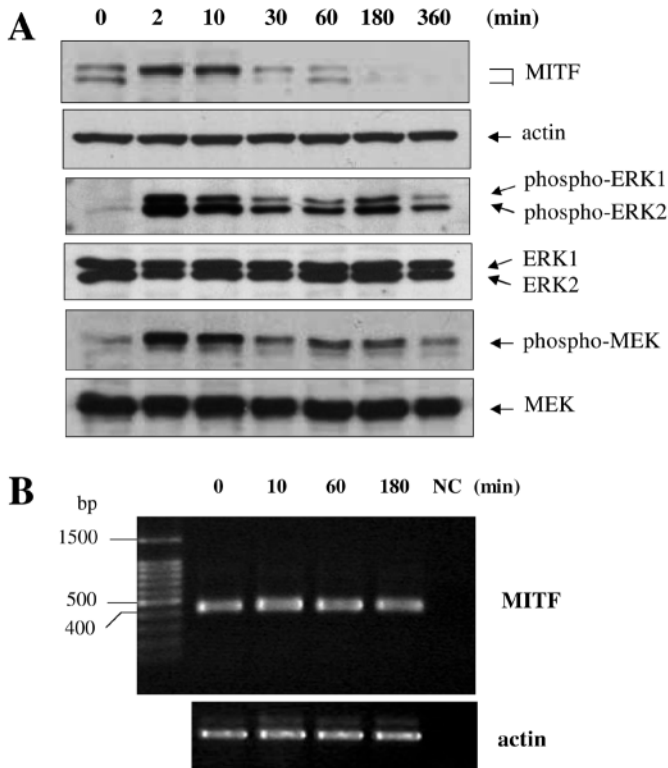

Fig. 3. S1P induces the degradation of MITF and stimulates the ERK signaling pathway. (A) After serum starvation, Mel-Ab cells were stimulated with 10 μM of S1P at the times indicated. Whole cell lysates were then subjected to western blot analysis with antibodies against MITF, phospho-specific ERK, and phospho-specific MEK. Equal protein loading was checked by reaction with actin, phosphorylation-independent ERK, and MEK antibodies. (B) RT-PCR analysis of MITF mRNA levels in S1P-stimulated cells. Cells were treated with S1P as in A. Total RNA was isolated from the cells and cDNA prepared. Equivalent amounts of cDNA were amplified with primers specific for MITF, and actin primers were used as a control to ensure the even loading of target cDNA. The resulting PCR products were analyzed by agarose gel electrophoresis. The lane on the left shows markers of the indicated size. NC, negative control.

Discussion

Despite our increasing knowledge of sphingolipids, the effects of sphingolipids on melanogenesis have received little attention. Because sphingolipids have been implicated in many biological processes, we were interested in their role on the regulation of melanogenesis. Recently, we reported that ceramide, a sphingolipid metabolite, inhibited melanin synthesis in Mel-Ab cells and human melanocytes (Kim et al., 2002; Kim et al., 2001). In the present study, we found that S1P inhibits melanin synthesis, and this reduction in melanogenesis by S1P was marked enough to be evident by observing cells under the phase contrast microscope. Moreover, S1P-induced hypopigmentation was found to be correlated with decreased tyrosinase activity, which regulates the rate-limiting step of melanin synthesis. Therefore, reduced tyrosinase activity may be responsible for the lower pigment content of S1P-treated cells. We compared the effect of S1P with that of kojic acid, since kojic acid is well known to affect

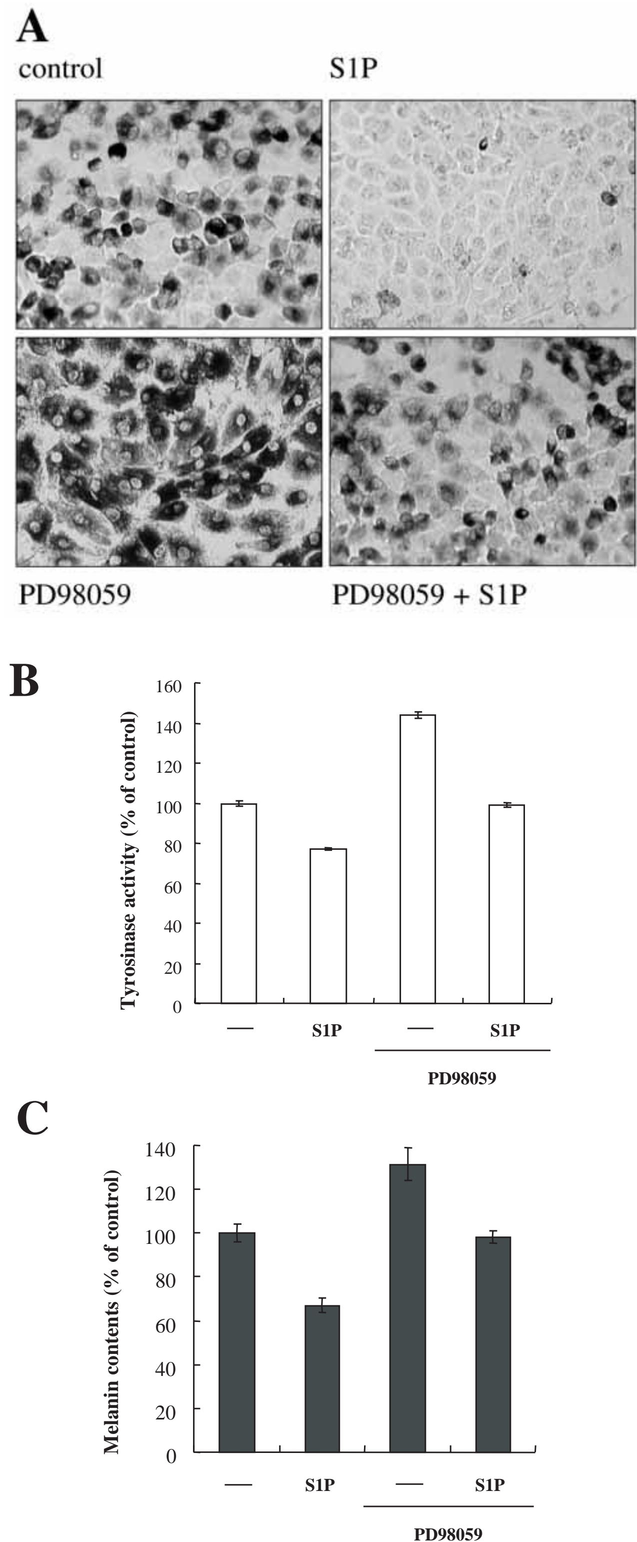

Fig. 4. Effects of S1P and PD98059 on melanogenesis in Mel-Ab cells. (A) Cells were cultured with 10 μM S1P and/or 20 μM PD98059 for 5 days, and phase contrast pictures were taken using a color video camera. The cells were pretreated with 20 μM of PD98059 for 1 hour and then cultured with 10 μM of S1P for 5 days, and tyrosinase activity (B) and melanin content (C) were measured, as described in Materials and Methods. Values indicate the mean of three independent experiments ± s.d.

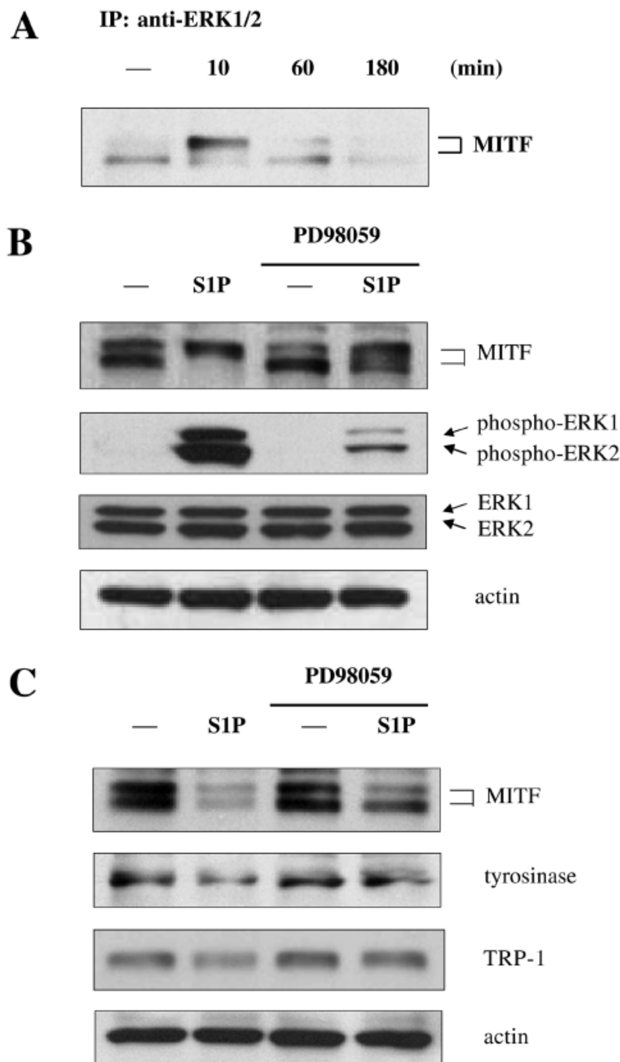

Fig. 5. S1P stimulation induces MITF degradation via the ERK signaling pathway. (A) After serum starvation, Mel-Ab cells were stimulated with 10 μM of S1P at the times indicated. Cell lysates were then immunoprecipitated with antibody against ERK1/2, and the MITF level was measured by immunoblotting. Cells were stimulated with 10 μM S1P for 10 minutes (B) or for 180 minutes (C) in the absence or presence of 20 μM PD98059. Whole cell lysates were then subjected to western blot analysis with antibodies against MITF, phospho-specific ERK, tyrosinase and TRP-1. Actin antibody was used a loading control.

melanin formation in melanocytes and melanoma cells (Mishima et al., 1988). Our results showed that the inhibitory effect of S1P was stronger than that of kojic acid. Interestingly, S1P did not alter tyrosinase activity in a cell-free system, whereas kojic acid directly suppressed tyrosinase. These relationships suggest that reduced pigmentation by S1P should be attributed to the action of S1P upon the signaling pathways regulating tyrosinase.

Several reports have indicated that the ERK signaling pathway is involved in the regulation of melanogenesis in melanocytes. It has been reported that UVA radiation-induced melanogenesis is associated with the activation of ERK in human melanocytes (Yanase et al., 2001). Moreover, the ERK signaling pathway is activated during cAMP-induced melanogenesis in B16 melanoma cells (Englaro et al., 1995). These studies suggest that the activation of ERK may increase melanin synthesis. However, PD98059, a specific inhibitor of the ERK pathway, increased the amount and the activity of tyrosinase. Moreover, the activation of the ERK pathway, and the presence of constitutive active mutants of Ras and MEK, led to the inhibition of tyrosinase gene transcription (Englaro et al., 1998). It was also reported that infection with the v-Ha-*ras* oncogene decreases melanogenesis in murine melanocytes (Tsukamoto et al., 1992). In a recent report, the inhibition of MEK activity with anthrax lethal toxin showed dramatic melanin production in human melanoma cells (Koo et al., 2002). In agreement with these studies, we also demonstrated that PD98059 augments melanin production in human melanocytes (Kim et al., 2002). In the present study, PD98059 treatment was also found to increase tyrosinase activity and melanin synthesis in Mel-Ab cells. These findings support our hypothesis that the inhibition of the ERK pathway induces melanin synthesis; in other words, the activation of the ERK pathway may be responsible for the inhibition of melanogenesis. Actually, S1P is a well-known lipid mediator that stimulates the ERK signaling pathway and then regulates cell proliferation (Cuvillier et al., 1996; Van Brocklyn et al., 2000). In our experiments, S1P clearly stimulated MEK and ERK activation and inhibited melanin synthesis in Mel-Ab cells, supporting our hypothesis. Interestingly, S1P strongly suppressed the proliferation of Mel-Ab cells (D.-S.K., unpublished), although the ERK pathway was markedly activated. However, it was also proposed that the kinetics of ERK are important in determining cellular response (Alblas et al., 1998). Therefore, the sustained activation of the ERK signaling pathway may play an important role in the regulation of melanogenesis and in the proliferation of Mel-Ab cells.

Previous studies have shown that the Akt signaling pathway is important in the regulation of melanogenesis in G361 melanoma cells (Oka et al., 2000) and that specific inhibition of the Akt pathway by LY294002 stimulates melanin synthesis in mouse B16 melanoma cells (Busca et al., 1996; Khaled et al., 2002). Therefore, we also examined whether the Akt signaling pathway is involved in melanin production in our cell system, because we found that S1P activates the Akt pathway in Mel-Ab cells (D.-S.K., unpublished). Thus, we examined the effect of LY294002, a phosphatidylinositol 3-kinase inhibitor, which blocks the Akt signaling pathway. We observed that LY294002 treatment increased melanin synthesis slightly, but that its effect was much less than that of PD98059 treatment (data not shown). These results indicate that the ERK pathway plays a critical role in the regulation of melanin synthesis, and that the activation of Akt by S1P may also contribute to the inhibition of melanogenesis. Thus, it appears that a complex network of signaling pathways, including the ERK pathway, may regulate melanogenesis. The involvement of the Akt pathway in melanogenesis remains to be elucidated.

MITF plays a critical role in melanogenesis, as the major transcriptional regulator of tyrosinase (Bentley et al., 1994; Busca and Ballotti, 2000; Tachibana, 2000). Decreased MITF gene expression is known to lead to the downregulation of melanocyte differentiation markers (Jimenez-Cervantes et al., 2001). Our results show that the activation of ERK after S1P

treatment is correlated with the phosphorylation and degradation of MITF. In accordance with reduced MITF, tyrosinase and TRP-1 protein were also reduced. Furthermore, PD98059 abolished the S1P-induced inhibition of tyrosinase activity and melanin synthesis and the phosphorylation and degradation of MITF. These results indicate that the inhibition of melanogenesis by S1P is probably due to the result of the stimulatory action of S1P on the ERK pathway. Recent studies have demonstrated that ERK phosphorylates MITF at serine 73 (Hemesath et al., 1998), and that the phosphorylation of MITF at serine 73 is responsible for MITF ubiquitination and degradation (Xu et al., 2000). Moreover, c-Kit activation triggered MITF degradation through the phosphorylation of MITF by ERK (Wu et al., 2000). In the present study, we also showed that PD98059 treatment prevented MITF phosphorylation and degradation. Thus, we suggest that S1P stimulates the ERK pathway and subsequently induces MITF phosphorylation and degradation via the activation of ERK, which leads to a reduced tyrosinase level and decreased melanogenesis.

Sphingosine was reported to inhibit protein kinase C (PKC) and is believed to be a negative regulator of cell signaling (Hannun and Bell, 1987). However, sphingosine stimulated the proliferation of quiescent 3T3 fibroblasts and Rat-1 fibroblasts (Gomez-Munoz et al., 1995; Zhang et al., 1990). In the present study, sphingosine showed a cytotoxic effect upon Mel-Ab cells, which may have been due to its detergent properties (Stoffel and Bister, 1973) or its inhibition of PKC (Merrill and Stevens, 1989). Moreover, DMS was found to potently inhibit sphingosine kinase and PKC (Igarashi et al., 1989). Thus, the inhibition of both sphingosine kinase and PKC by DMS may explain its strong cytotoxic effect.

In summary, we demonstrate for the first time the hypopigmenting effect of S1P. Moreover, our results show that increased ERK activation by S1P leads to MITF phosphorylation and degradation, which in turn are responsible for decreased melanin synthesis.

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