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Research Paper

Sphingosylphosphorylcholine inhibits melanin synthesis via pertussis toxin-sensitive MITF degradation

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Abstract

Objectives Sphingolipids act as structural components in cell membranes, and form lipid intermediates that have functional roles as signalling molecules in various cellular processes. Our previous findings have suggested that sphingolipid metabolites are deeply involved in the regulation of melanogenic processes. In this study we aimed to examine sphingosylphosphorylcholine-mediated signalling pathways related to melanogenesis.

Methods We determined the hypopigmenting effects and the related signalling pathways of sphingosylphosphorylcholine in Mel-Ab cells. In particular, we analysed the involvement of the G-protein-coupled receptor in sphingosylphosphorylcholine-induced MITF degradation.

Key findings Western blotting revealed that sphingosylphosphorylcholine induced the activation of extracellular signal-regulated kinase (ERK), as well as Akt. Moreover, the specific Akt pathway inhibitor LY294002 blocked the hypopigmenting effect of sphingosylphosphorylcholine and abrogated the sphingosylphosphorylcholine-mediated down-regulation of microphthalmia-associated transcription factor (MITF), showing that the Akt pathway is involved in sphingosylphosphorylcholine-mediated melanin inhibition. Treatment with the proteasome inhibitor MG132 blocked the decrease in MITF by sphingosylphosphorylcholine, but sphingosylphosphorylcholine did not decrease the level of MITF mRNA, indicating that the reduction in the level of MITF results from MITF degradation. Furthermore, pre-incubation of Mel-Ab cells with pertussis toxin completely abolished the hypopigmenting effects and the activation of ERK and Akt by sphingosylphosphorylcholine, suggesting that the effects of sphingosylphosphorylcholine are mainly dependent on the G-protein-coupled receptor).

Conclusions Together, these results suggest that sphingosylphosphorylcholine reduces melanin synthesis via pertussis toxin-sensitive ERK and Akt activation, and subsequent MITF degradation.

Keywords Akt; melanocytes; microphthalmia; sphingosylphosphorylcholine; tyrosinase

Introduction

It is well known that sphingolipids act as structural components in cell membranes, and form lipid intermediates that have functional roles as signalling molecules in various cellular processes.[1,2] In previous studies, we have attempted to elucidate the role of sphingolipids in melanogenesis in melanocytes. Thus, we have shown that a sphingolipid metabolite, ceramide, regulates melanin synthesis in human melanocytes and Mel-Ab cells,[3,4] and that another sphingolipid (sphingosine-1-phosphate) and the structurally related lysophosphatidic acid also decrease melanin synthesis.[5,6] From these findings, we have suggested that sphingolipid metabolites are deeply involved in the regulation of melanogenic processes.

The sphingomyelin metabolite sphingosylphosphorylcholine (SPC) is generated by N-deacylation of sphingomyelin, and has very recently become the subject of interest and controversy.[2] SPC has cell type-specific functions in major tissues. In terms of melanogenesis, it has been reported that SPC stimulates melanogenesis in human melanocytes.[7] Furthermore, the same authors suggested that stimulation of melanogenesis by SPC was associated with a marked increase in the phosphorylation of extracellular signal-regulated kinase (ERK). In contrast, we recently reported that SPC-induced ERK

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activation inhibits melanin synthesis in human melanocytes.[8] Thus, in this study, we have further examined SPC-mediated signalling pathways related to melanogenesis. Although many studies have attempted to identify a high-affinity SPC-specific membrane receptor, no receptors have been conclusively identified.[2] Therefore, it remains to be determined whether SPC might regulate intracellular pathways via membrane receptors, or act as a direct intracellular messenger in melanogenesis of melanocytes.

In mammalian melanocytes, tyrosinase plays a critical role in the process of melanogenesis because it catalyses the two rate-limiting steps of melanogenesis.[9] Thus, melanin production is mainly dependent on tyrosinase expression and activation.[10] Furthermore, microphthalmia-associated transcription factor (MITF) strongly regulates the expression of tyrosinase, indicating that MITF is an important transcriptional regulator in the melanogenic process.[11–14]

It has been suggested that the ERK signalling pathway is involved in melanogenesis.[15] ERK activation causes MITF phosphorylation at serine 73, which is followed by MITF ubiquitination and degradation.[16–18] Furthermore, a 90 kDa ribosomal S6 kinase (RSK-1) has been shown to phosphorylate MITF at serine 409, and this phosphorylation is followed by MITF degradation.[17] On the other hand, the Akt signalling pathway is also involved in the regulation of melanogenesis in G361 melanoma cells[19] and specific inhibition of the Akt pathway by LY294002 stimulates melanin synthesis in mouse B16 melanoma cells.[20,21]

Therefore, we have hypothesized that the ERK pathway, as well as the Akt pathway activated by SPC, participates in melanogenesis. In this study, we determined the hypopigmenting effects and the related signalling pathways of SPC in Mel-Ab cells. In particular, we analysed the involvement of the G-protein-coupled receptor (GPCR) in SPC-induced MITF degradation.

Materials and Methods

Reagents

The reagents were purchased from the following sources: SPC (Avanti Polar Lipids, Alabaster, USA); pertussis toxin (PTX; Alexis, San Diego, USA); LY294002 and MG132; (Calbiochem, San Diego, USA); fetal bovine serum (FBS; Hyclone, Logan, USA); protease inhibitors (CompleteTM; Roche, Mannheim, Germany); enhanced chemiluminescence plus kit (Amersham International, Little Chalfont, UK), RNeasy Mini Kit (Qiagen, Valencia, USA); ImProm II Reverse Transcription System (Promega, Madison, USA); and 12-*O*-tetradecanoylphorbol-13-acetate (TPA), cholera toxin (CT), fatty acid-free bovine serum albumin (BSA), Triton X-100, Tris, β-mercaptoethanol, phenylmethylsulfonyl fluoride, synthetic melanin and levodopa (L-DOPA; Sigma, St Louis, USA). The antibodies used in this study were purchased from the following sources: phospho-specific Akt (Ser473, No. 9271S), phospho-specific RSK-1 (Thr359/Ser363, No. 9344S), phospho-specific ERK1/2 (Thr202/Tyr204, No. 9101S), total (phosphorylated and non-phosphorylated) ERK1/2 (No. 9102), phospho-specific MEK (Ser217/221, No. 9121) and total MEK (No. 9122) were from Cell Signaling Technology (Beverly, USA); microphthalmia Ab-3 (C5+D5, MS-773-P0; NeoMarkers, Fremont, USA); total RSK-1 (C-21, sc-231), total Akt (C-20, sc-1618), tyrosinase (C-19) and actin (I-19) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, USA); phospho-serine (61-8100) was from Zymed (San Francisco, USA); and phospho-specific MITF (Ser73) was kindly provided by Dr D.E. Fisher (Harvard Medical School, Boston, USA).

Cell cultures

The Mel-Ab cell line used in this study is a mouse-derived spontaneously immortalized melanocyte cell line, which produces large amounts of melanin.[22] Mel-Ab cells were incubated in DMEM supplemented with 10% FBS, 100 nM TPA, 1 nM CT, 50 μg/ml streptomycin and 50 μg/ml penicillin at 37°C in 5% CO_2.

Measurement of melanin content and microscopic examination

Melanin content was measured as described previously,[23] with slight modification. Briefly, Mel-Ab cells were treated with SPC for four days. Cell pellets were dissolved in 1 ml of 1 M NaOH at 100°C for 30 min and centrifuged for 20 min at 16 000*g*. The optical density (OD) of the supernatants was measured at 400 nm using an ELISA reader. Standard curves of synthetic melanin (0–300 μg/ml) were prepared in triplicate for each experiment. Before measuring the melanin content, Mel-Ab cells were observed under a phase contrast microscope (Olympus Optical Co., Tokyo, Japan) and photographed using a $CoolSNAP_{cf}$ digital video camera system (Roper Scientific Inc., Tucson, USA) supported by RS Image software (Roper Scientific, Inc.).

Tyrosinase activity

Tyrosinase activity was determined, as previously described,[20] with slight modification. Briefly, Mel-Ab cells were cultured in six-well plates. After incubating with SPC for four days, the cells were 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 were clarified by centrifugation at 10 000*g* for 5 min. After quantifying the protein levels and adjusting the protein concentrations with 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 then added to each well. The control wells contained 90 μl of lysis buffer and 10 μl of 10 mM L-DOPA. After incubation at 37°C for 20 min, dopachrome formation was monitored by measuring absorbance at 475 nm using an ELISA reader. Tyrosinase activity was expressed as the percentage of non-treated controls.

Western blot analysis

Mel-Ab 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), 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 an enhanced chemiluminescence plus kit (Amersham International).

Immunoprecipitation assay

Cells were grown in 100 mm culture dishes, starved of serum for 24 h, treated with SPC as indicated, lysed on ice for 10 min 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-glycerol phosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride) and centrifuged at 15 000 rev/min for 10 min at 4°C in a microcentrifuge. The lysates were immunoprecipitated using an antibody against MITF and protein A agarose beads, which were washed three times with cell lysis buffer to eliminate non-specific binding. The level of phosphoserine was measured by immunoblotting.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the cells using an RNeasy Mini Kit (Qiagen). Then, 1 μg of RNA was reverse transcribed using an ImProm II Reverse Transcription System (Promega). The cDNA obtained was amplified with the following primers: MITF (366 bp product) forward 5′-TCCGTCTCTCACTGGATTGGTG-3′ and reverse 5′-CGTGAATGTGTGTTCATGCCTGG-3′; and tyrosinase (568 bp product) forward 5′-CTCCGCTGGCCATTTCCCTA-3′ and reverse 5′-GGTGCTTCATGGGCAAAATC-3′. The PCR conditions were 30 cycles for 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, and the resulting PCR products were visualized by electrophoretic separation on 1.5% agarose gels with ethidium bromide staining. Specific primers for GAPDH were added as a control.

Results

Effects of sphingosylphosphorylcholine on tyrosinase activity and melanin synthesis

We previously reported that SPC inhibits melanin synthesis in normal human melanocytes.[8] To confirm the effect of SPC on melanogenesis, Mel-Ab cells were treated with SPC at concentrations of 0.1–10 μM. Four days after SPC treatment, we determined tyrosinase activity and found that SPC significantly reduced tyrosinase activity in a concentration-dependent manner (data not shown). Furthermore, SPC significantly reduced the melanin content of Mel-Ab cells (data not shown). SPC was not cytotoxic to Mel-Ab cells in the concentration range 0.1–10 μM (data not shown).

Sphingosylphosphorylcholine activates the ERK and the Akt pathways and reduces MITF protein levels

SPC induces ERK activation and MITF down-regulation in human melanocytes.[8] Thus, we confirmed that SPC induced the activation of MEK (MAPK/ERK kinase), ERK and RSK-1 in Mel-Ab cells (Figure 1a). Moreover, SPC was able to activate the Akt pathway, with a peak occurring 10 min after SPC treatment (Figure 1a).

It has been reported that activation of ERK and RSK-1 is linked to MITF phosphorylation at serines 73 and 409, respectively.[16,17] However, in this study we did not detect phosphorylation of serine 73, although ERK was clearly activated (Figure 1a). Furthermore, we attempted to identify

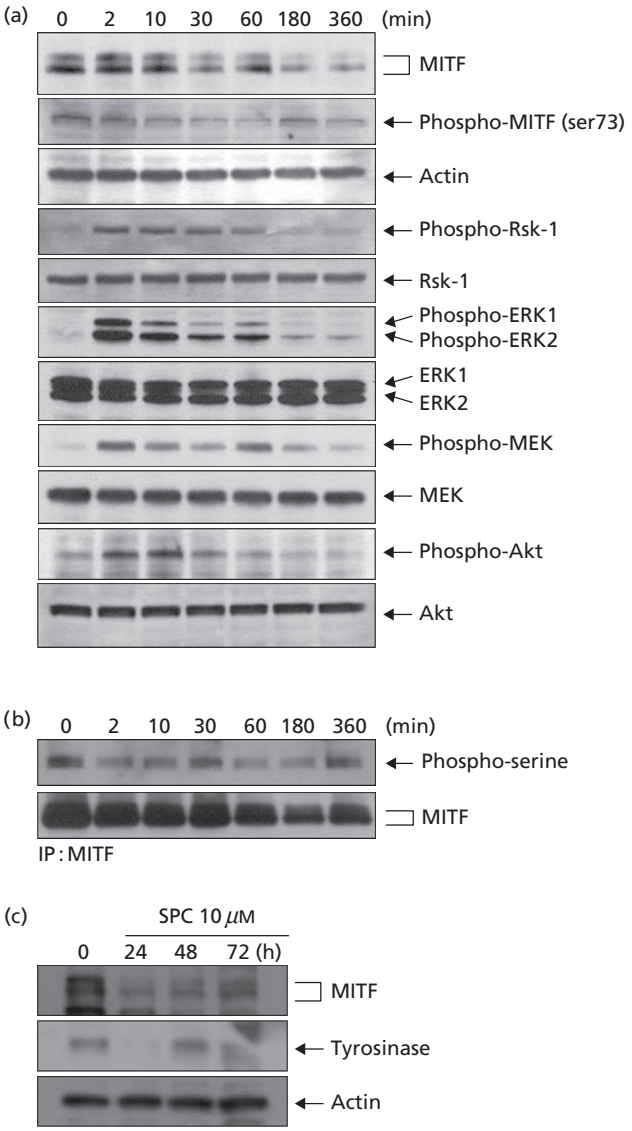

Figure 1 Sphingosylphosphorylcholine stimulates the ERK and Akt pathways and down-regulates MITF and tyrosinase. After serum starvation, Mel-Ab cells were stimulated with 10 μM sphingosylphosphorylcholine (SPC) for the times indicated. (a) Whole cell lysates were then subjected to Western blot analysis with phospho-specific antibodies. (b) Otherwise, cell lysates were immunoprecipitated with antibody against MITF, and the phosphoserine level was measured by immunoblotting. (c) Cells were cultured with 10 μM SPC for 24, 48 or 72 h. Whole cell lysates were subjected to Western blot analysis with antibodies against MITF and tyrosinase.

MITF phosphorylation at other serine positions using a phosphoserine specific antibody. However, we found that serine phosphorylation decreased slightly (Figure 1b). Thus, we suggest that SPC does not induce MITF phosphorylation, although MITF protein was clearly down-regulated 3 h after SPC treatment (Figure 1a).

We next examined the protein levels of MITF and tyrosinase after SPC treatment in long-term cultures. The cells were continuously kept in the medium with SPC for 72 h. As shown in Figure 1c, the MITF levels were clearly reduced 24 h after SPC treatment; tyrosinase levels also decreased according to MITF reduction. After the initial drop, MITF and tyrosinase protein levels began to recover 48 h after SPC treatment. These results suggest that SPC decreases melanin synthesis by down-regulating MITF and tyrosinase (Figure 1c).

Inhibition of the Akt pathway by LY294002 restored sphingosylphosphorylcholine-induced hypopigmentation

As we have shown in human melanocytes,[8] the involvement of ERK activation by SPC in melanogenesis of Mel-Ab cells was confirmed and the same results were obtained (data not shown). Since SPC induced the activation of Akt (Figure 1a), Mel-Ab cells were treated with SPC for four days in the absence or presence of LY294002, a specific Akt pathway inhibitor, then photographed under a phase contrast microscope (Figure 2a). LY294002 abolished the inhibition of melanin synthesis and MITF down-regulation by SPC (Figure 2a, b). We next showed that LY294002 blocked Akt activation by SPC (Figure 2b). Therefore, these results suggest that the Akt pathway is also involved in SPC-mediated MITF down-regulation.

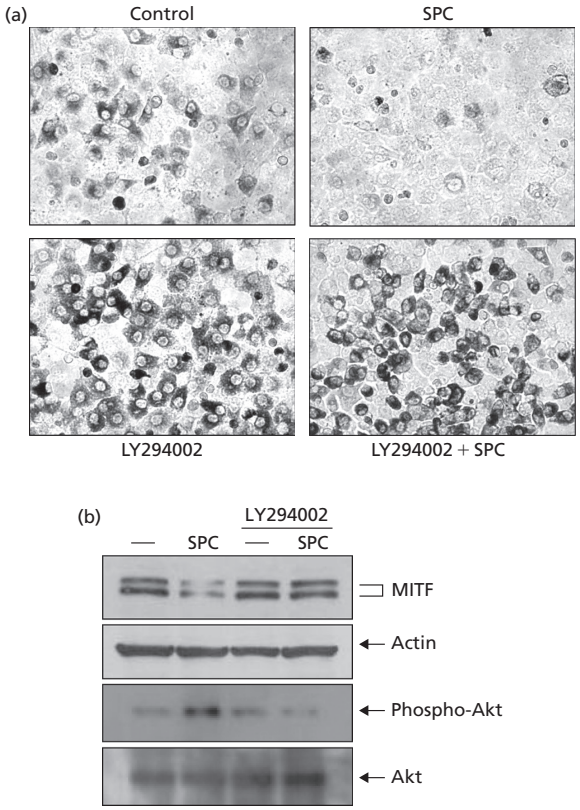

Figure 2 Effect of sphingosylphosphorylcholine and LY294002 on melanogenesis. Mel-Ab cells were pretreated with 20 μM of LY294002 for 10 min and then cultured with 10 μM sphingosylphosphorylcholine (SPC) for four days. (a) Phase contrast photographs were taken using a digital video camera. (b) After serum starvation, cells were cultured with 10 μM SPC for 3 h in the presence or absence of 20 μM LY294002. Whole cell lysates were then subjected to Western blot analysis with antibodies against phospho-specific Akt or MITF.

Down-regulation of MITF level is a result of MITF degradation

We next determined whether MITF down-regulation by SPC is a result of the decrease in MITF mRNA expression or MITF degradation. Thus, RT-PCR assays using specific primers for MITF and tyrosinase were performed. As shown in Figure 3a, the expression of MITF mRNA and tyrosinase was not changed by SPC treatment until 6 h. To determine whether MITF instability was involved in proteasome-mediated degradation, we examined the levels of MITF

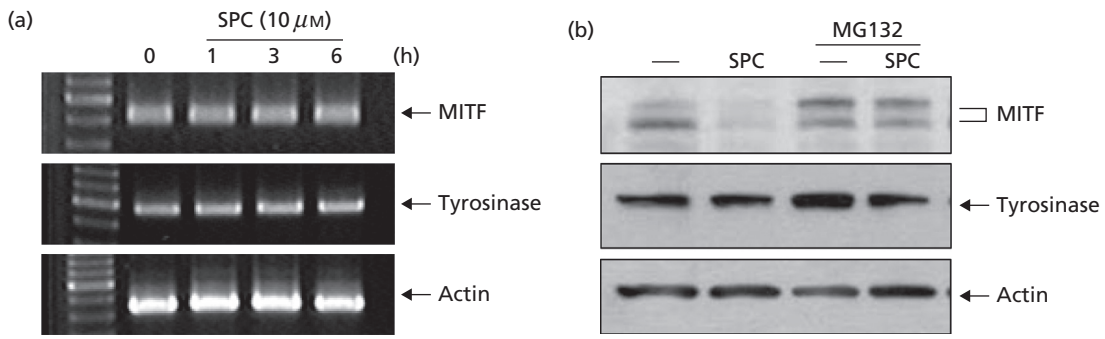

Figure 3 Effect of sphingosylphosphorylcholine on MITF expression and degradation. (a) After serum starvation, Mel-Ab cells were cultured with 10 μM of sphingosylphosphorylcholine (SPC) for 1–6 h. RT-PCR analysis of MITF and tyrosinase mRNA levels was then performed as described in Materials and Methods. Equivalent amounts of cDNA were amplified with primers specific for MITF or tyrosinase, and actin primers were used as a control to ensure the even loading of the target cDNA. The resulting PCR products were analysed by agarose gel electrophoresis. (b) After serum starvation, Mel-Ab cells were pretreated with 25 μM of MG132 for 1 h and then cultured with 10 μM SPC for 3 h. Whole cell lysates were subjected to Western blot analysis with antibodies against MITF and tyrosinase.

protein and tyrosinase 3 h after SPC treatment with or without the proteasome inhibitor, MG132. As shown in Figure 3b, the MITF levels were clearly reduced 3 h after SPC treatment; however, the tyrosinase levels were not changed. Of note, MG132 almost prevented SPC-induced MITF down-regulation. These results suggest that SPC decreases melanin synthesis by increased MITF degradation.

Sphingosylphosphorylcholine exerts hypopigmenting effects in a pertussis toxin-sensitive manner

To examine whether the hypopigmenting effects of SPC is mediated through specific receptors, we investigated the effects of SPC in the presence of PTX, which inactivates the α-subunit of Gi/Go. Mel-Ab cells were treated with SPC for four days in the presence or absence of PTX and then photographed under a phase contrast microscope (Figure 4a).

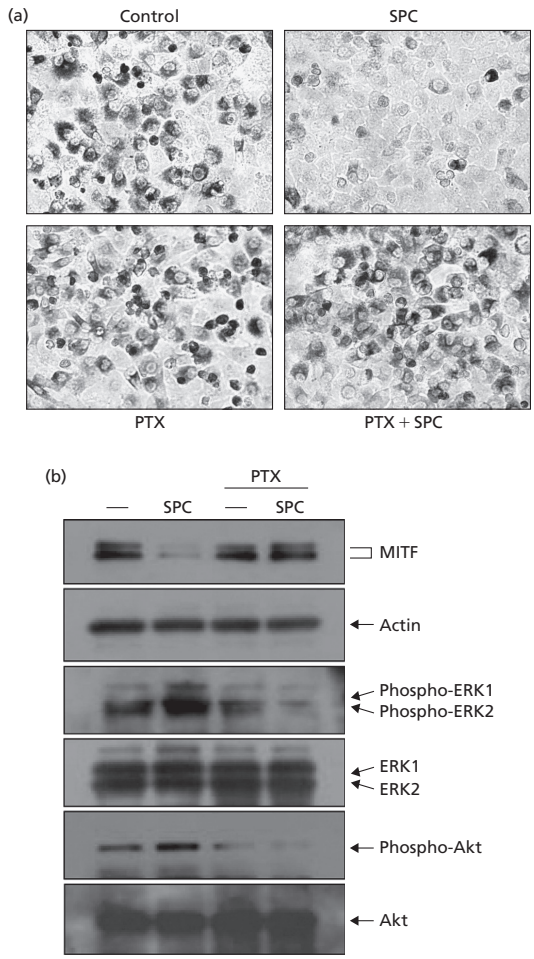

Figure 4 Involvement of G-protein-coupled receptor in sphingosylphosphorylcholine-mediated hypopigmentation. Mel-Ab cells were pretreated with 100 ng/ml of pertussis toxin (PTX) for 3 h and then cultured with 10 μM sphingosylphosphorylcholine for four days. (a) Phase contrast photographs were taken using a digital video camera. (b) After serum starvation, cells were cultured with 10 μM SPC for 3 h in the presence or absence of 100 ng/ml of PTX. Whole cell lysates were then subjected to Western blot analysis with antibodies against MITF, phospho-ERK, or phospho-Akt.

Pre-incubation of cells with PTX almost completely abolished the inhibition of melanin synthesis and MITF down-regulation by SPC (Figure 4a, b). Furthermore, we showed that PTX blocked ERK and Akt activation induced by SPC (Figure 4b). Together, these results indicate that GPCR is also involved in SPC-mediated signalling pathways and the resulting hypopigmentation.

Discussion

We have suggested that sphingolipids are involved in the regulation of melanogenesis.[4–6] Although Higuchi *et al.*[7] interpreted ERK activation as a signal for the up-regulation of melanogenesis, they did not find direct evidence that SPC increases melanin levels. In this study, we confirmed that ERK activation suppresses melanogenesis, as we previously suggested.[4,5] In addition, Higuchi *et al.*[7] showed an increase in MITF mRNA at only one time point (24 h). These differences may simply be due to the time points used.

It has been reported that ERK and RSK-1 activation leads to MITF phosphorylation at serines 73 and 409, respectively.[17,18] Previously, we reported that sphingosine-1-phosphate (S1P) induces ERK activation and MITF phosphorylation at serine 73.[5] However, in this study, serine 73 was not phosphorylated by SPC, although ERK was clearly activated. Further results showed that MITF was not phosphorylated at serine by SPC. These findings indicate that ERK activation does not always phosphorylate MITF, and that MITF can be down-regulated without being phosphorylated. Although SPC did not lead to MITF phosphorylation, our results show that PD98059 restored MITF down-regulation by SPC,[8] indicating that SPC-induced MITF down-regulation was mediated by the ERK pathway. To clarify the differences in the signalling pathways and MITF down-regulation between S1P and SPC, further investigation is needed.

It has also been reported that the Akt pathway is involved in melanogenesis.[19,21] It is known that Akt phosphorylates glycogen synthase kinase 3β (GSK3β) at serine 9, leading to GSK3β inactivation. Furthermore, GSK3β has been shown to phosphorylate MITF at serine 298, thereby enhancing the binding of MITF to the tyrosinase promoter.[24] From these reports, it can be postulated that activation of Akt leads to inhibition of MITF phosphorylation at serine 298, and subsequently suppression of melanin production. Thus, we found decreased MITF phosphorylation after SPC treatment (Figure 1b). With respect to the Akt pathway, we showed that LY294002 treatment blocked not only SPC-induced MITF degradation, but also completely blocked hypopigmentation. These results indicate that the activation of Akt by SPC also contributes to the inhibition of melanogenesis. Thus, it appears that a complex network of signalling pathways, including the ERK and Akt pathways, may regulate melanogenesis.

It has also been reported that peroxisome proliferator-activated receptor (PPAR) alpha activation induces down-regulation of Akt and decreases metastatic activity of melanoma cells.[25] These findings indicate that the Akt pathway is deeply involved in melanoma metastasis. In this study, SPC led to the activation of Akt, which may increase melanoma metastasis. Furthermore, cutaneous malignant

melanomas exhibit different levels of pigmentation.[26] Thus, further investigation is needed to determine the effects of SPC-induced Akt activation in the progression of melanoma via regulation of melanogenesis.

Our results showed that SPC-induced ERK and Akt activation was blocked by PTX, suggesting that PTX-sensitive GPCR is involved in SPC-mediated hypopigmentation. In recent years, a number of studies have attempted to identify a high affinity SPC-specific membrane receptor. Initially, it was reported that ovarian cancer G-protein-coupled receptor 1 (OGR1) bound SPC with high affinity and specificity.[27] In addition, GPR4, another GPCR, was reported to bind SPC with high affinity;[28] however, this work was recently retracted due to a lack of reproducibility.[29] Thus, no SPC receptor has been conclusively identified.[2] In contrast, it has been reported that SPC is a selective agonist for S1P receptors.[30] However, our results have shown that SPC and S1P have different effects, such as MITF phosphorylation at serine 73, suggesting that a distinct membrane receptor for SPC may exist.

Conclusions

ERK and Akt activation by sphingosylphosphorylcholine leads to MITF down-regulation, which in turn reduces melanin synthesis. In addition, SPC-induced MITF down-regulation appears to be caused by MITF degradation. Furthermore, not only ERK and Akt activation, but also MITF down-regulation, is PTX-sensitive. Based on these results, we propose that SPC inhibits melanin synthesis via GPCR-mediated ERK and Akt activation and subsequent MITF degradation.

Declarations

Conflict of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

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