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Activation of Akt/PKB at Serine 473 by N-acetylphytosphingosine (NAPS) and C_2-ceramide Reduces Melanin Synthesis in B16F10 Mouse Melanoma Cells

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Abstract

Sphingolipid metabolites regulate many aspects of cell proliferation, differentiation, and apoptosis. In the present study, we have assessed the effects of the novel phytosphingosine derivative, N-acetylphytospingosine (NAPS), on the depigmentation of murine B16F10 melanoma cells, and have also attempted to identify the possible signaling pathway involved, in comparison with C_2-ceramide. NAPS and C_2-ceramide both inhibited the growth of the B16F10 cells in a dose-dependent manner. Melanin content and tyrosinase activity were significantly reduced in response to treatment with NAPS and C_2-ceramide at concentrations in a range between 1-5 μM. However, the levels of tyrosinase mRNA, as well as the levels of tyrosinase related protein-1 (TRP-1) and tyrosinase related protein-2 (TRP-2) genes and the level of tyrosinase protein remained unaffected by treatment with either NAPS or C_2-ceramide. We also attempted to determine the signaling pathway exploited by NAPS and C_2-ceramide. Interestingly, the phosphorylation of Akt/PKB at serine 473 by NAPS was reduced at the 5 minute mark, whereas C_2-ceramide induced the phosphorylation of Akt/PKB at serine 473. Finally, Akt/PKB activity in the NAPS-treated cells was elevated in comparison with the untreated cells. LY294002, a specific PI3-K inhibitor which is located upstream of Akt/PKB, inhibited the phosphorylation of Akt/PKB, but induced an increase in melanin synthesis. These results suggest that the activation of Akt/PKB at serine 473 is related with the suppression of melanin production in the B16F10 mouse melanoma cells. Therefore, the mechanisms exploited by NAPS and C_2-ceramide responsible for the depigmentation of B16F10 cells were concluded to involve the inhibition of melanosomal tyrosinase activity.

Keywords: N-acetylpytosphingosine (NAPS), C_2-ceramide, Akt/PKB, melanogenesis, B16F10

Sphingolipid metabolites, including ceramide and sphingosine, are believed to play important roles in the regulation of cellular activities[1]. Ceramide regulates cell proliferation, differentiation, and apoptosis[2], and sphingosine also inhibits cell growth and induces apoptosis in a variety of cell types[3,4]. Interestingly, cellular responses to ceramide depend on the particular cell types used. Indeed, ceramide stimulates cell proliferation in confluent quiescent Swiss 3T3 fibroblasts[5], but inhibits the growth of proliferating normal fibroblasts[6]. Ceramide has also been shown to induce differentiation in DJM-1 keratinocyte-related cells[7]. Phytosphingosine (PS) is structurally similar to both sphingosine and ceramide. It has been reported that both PS and the noble PS derivative, N-acetyl-phytosphingosine (NAPS) (Fig. 1), induce cell death in Chinese hamster cells[8]. Nevertheless, a few groups have reported on the effects of ceramide on melanocytes[9,10], although the cellular functions of the PS derivatives have yet to be adequately clarified. Therefore, we have attempted to delineate the effects of cell-permeable ceramide and NAPS on the regulation of melanogenesis in B16F10 melanoma cells.

Melanin is a mixture of pigmented bipolymers, which is synthesized by both melanocytes and melanoma cells. Melanin synthesis is regulated via the activities of melanogenic enzymes, such as tyrosinase, tyrosinase-related protein-1 (TRP-1), and tyrosinase-related protein-2 (TRP-2). Tyrosinase is a bifunctional enzyme, and plays a pivotal role in the modulation of melanin production[11]. The initial step of melanin synthesis begins with involves the oxidation of tyrosine, which is catalyzed by tyrosinase. TRP-1 and TRP-2 operate at downstream points in melanin synthesis[12]. Melanogenesis has been shown to be modulated by a variety of intracellular signaling mechanisms, including protein kinases such as cAMP -dependent protein kinase[13], protein kinase C-α (PKC

-α)[14], protein kinase C-β (PKC-β)[15], and mitogen-activated protein kinase (MAPK)[16]. The activation of Akt/protein kinase B (Akt/PKB) appears to be sufficient for the suppression of melanin production in G361 melanoma cells[17]. In some other fashion, Akt/PKB is also phosphorylated by several growth factors, including platelet-derived growth factor and insulin, for cell survival and growth[18], and its activation occurs via a pathway involving PI3-K[19,20]. Furthermore, C_2-ceramide has been shown to inhibit the proliferation of human melanocytes via the inactivation of Akt/PKB[9,10].

In this study, we have utilized the noble phytosphingosine derivative, NAPS, and have evaluated its effects on melanogenesis in B16F10 melanoma cells, in comparison to C_2-ceramide. We measured melanin production and tyrosinase activity elicited by NAPS and C_2-ceramide, as well as the expression of tyrosinase, TRP-1, and TRP-2 mRNA in the B16F10 melanoma cells. We also attempted to delineate the Akt/PKB-associated signaling pathway exploited by NAPS in the modulation of melanogenesis, in comparison with that exploited by C_2-ceramide.

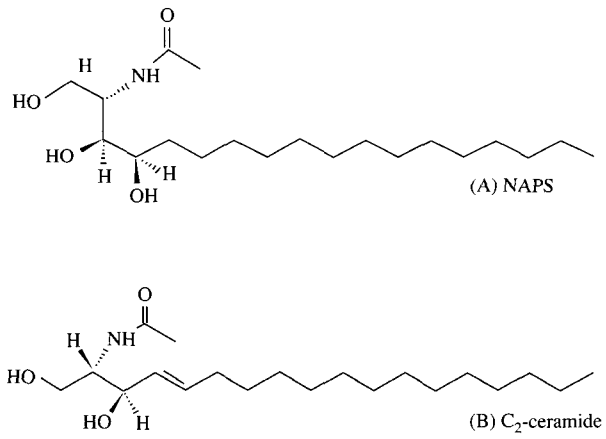

Fig. 1. The structure of NAPS and C_2-ceramide.

NAPS and C_2-ceramide Inhibit Cell Growth

In order to evaluate the effects of NAPS and C_2-ceramide on cell growth, the cells were cultured with NAPS and C_2-ceramide at a concentration range between 1-5 μM for 72 hours, after which MTT assays were conducted, as is described in the Materials and Methods section. Our results indicated that both NAPS and C_2-ceramide inhibited the growth of the B16F10 cells in a dose-dependent manner (Fig. 2). At a concentration of 5 μM, NAPS and C_2-ceramide both exhibited inhibitory effects commensurate with a 20-25% reduction in cell growth.

NAPS and C_2-ceramide Suppressed Tyrosinase Activity and Melanin Synthesis

Melanogenesis begins with the catalysis of the hydroxylation of tyrosine by tyrosinase, which is the principal enzyme in the regulation of melanogenesis. We measured the tyrosinase activity and melanin contents in the B16F10 cells treated with NAPS and C_2-ceramide at a range of concentration between 1-5 μM. NAPS and C_2-ceramide were shown to significantly suppress tyrosinase activity in a dose-dependent manner (Fig. 3A). The melanin content of the B16F10 cells was also reduced, to 29-31% as compared to the control group (Fig. 3B). Therefore, the activity of tyrosinase was modulated by NAPS and C_2-ceramide in a manner strongly consistent with the observed regulatory effects on melanin production.

NAPS and C_2-ceramide did not Alter Levels of Tyrosinase mRNA and Protein

Tyrosinase activity and melanin synthesis were reduced in response to NAPS and C_2-ceramide treatment, as is shown in Figure 3. We also attempted to determine whether NAPS and C_2-ceramide could also modulate the levels of tyrosinase and TRP-1 and TRP -2 mRNA, all of which are important downstream of tyrosinase. Therefore, we attempted to determine the mRNA expression levels of tyrosinase, TRP-1, and TRP-2, via semi-quantitative RT-PCR. NAPS or C_2-

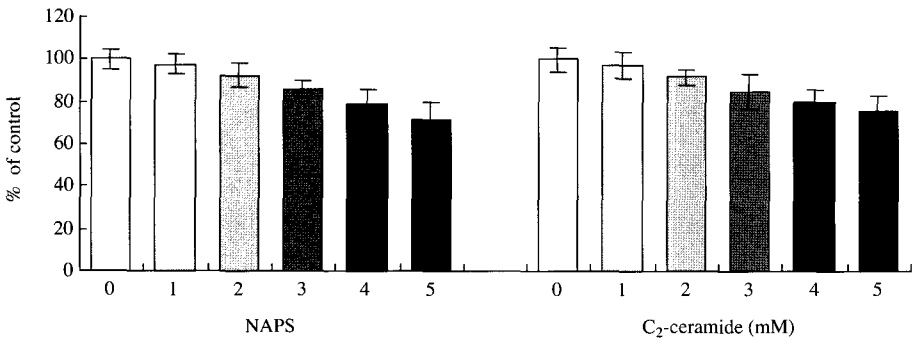

Fig. 2. NAPS and C_2-ceramide inhibited the proliferation of B16F10 cells. Cells were treated with 1-5 μM NAPS or C_2-ceramide for 3 days, as described in the Materials and Methods section. Cell proliferation is expressed as a percentage of the control cells (100%) in serum-free medium. The values are expressed as means±S.D. (n=4).

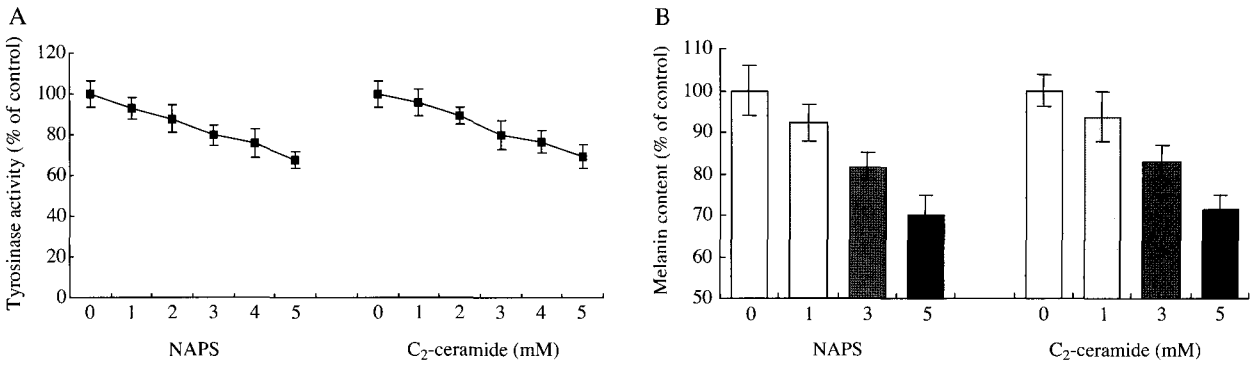

Fig. 3. (A) Tyrosinase activity was reduced in the B16F10 cells by treatment with NAPS and C_2-ceramide. (B) NAPS and C_2-ceramide inhibited melanin synthesis in the B16F10 cells Cells were cultured for 3 days with 1-5 μM NAPS or C_2-ceramide. Tyrosinase activity and melanin content were measured as described in the Materials and Methods section. The results are expressed as a percentage of the control cells (100%). Results are the average of three independent experiments ± S.D.

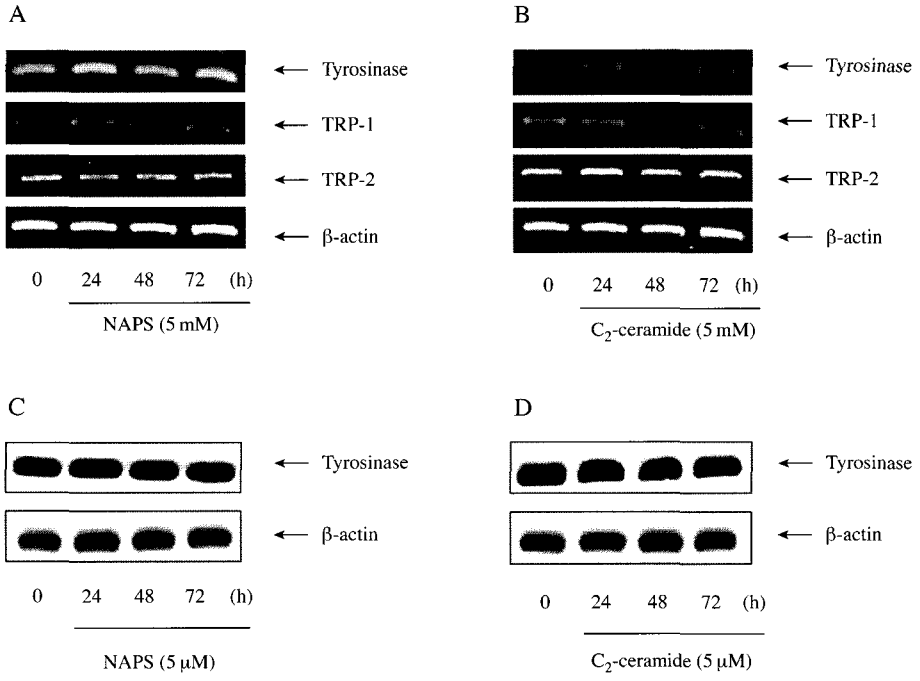

Fig. 4. Levels of tyrosinase mRNA, TRP-1 and TRP-2 mRNA, and tyrosinase protein in NAPS (A, C) and C_2-ceramide (B, D)-treated B16F10 melanoma cells. Cells were cultured with 5 μM NAPS or 5 μM C_2-ceramide for 24-72 hours. Total RNA was extracted using TRIzol, and RT-PCR analysis was used to determine the expression levels of tyrosinase, TRP-1 and TRP-2 mRNA induced by the 5 μM NAPS (A) and 5 μM C_2-ceramide (B). Protein extracts were also harvested from 5 μM NAPS (C) and 5 μM C_2-ceramide (D) treated cells, separated by SDS-PAGE, and subjected to Western blot analysis using either tyrosinase or β-actin antibodies.

ceramide (5 μM) were treated for 24, 48, and 72 hours. As is shown in Figure 4A and 4B, we noted no alterations in the mRNA levels of tyrosinase, TRP-1, or TRP-2. In order to evaluate the effects of NAPS or C_2-ceramide on the expression level of tyrosinase protein, we conducted Western blotting assays. However, neither NAPS nor C_2-ceramide altered the levels of tyrosinase protein in the cells (Fig. 4C, 4D). Accordingly, we suggest that both NAPS and C_2-ceramide could inhibit tyrosinase activity at the melanosomal level.

Increased Akt/PKB Phosphorylation by NAPS and C_2-ceramide Treatment

To determine the signaling pathways exploited by NAPS and C_2-ceramide, we conducted Western blotting assays using phosphor-Akt/PKB antibody. The cells were cultured in a medium containing 5 μM of either NAPS or C_2-ceramide. Akt/PKB phosphorylation was notably increased, in a time-dependent fashion, in response to C_2-ceramide treatment, and this effect persisted for 7 hours. NAPS treatment induced the activation of Akt/PKB at 1 hour post-treatment, and this effect gradually became more pronounced

until 7 hours post-treatment (Fig. 5). LY294002, a specific PI3-K inhibitor, which blocks the Akt signaling pathway, was shown to inhibit NAPS and C_2-ceramide-induced Akt/PKB phosphorylation at serine 473 in the B16F10 cells, as is shown in figure 6. This indicates that the inhibition of the Akt pathway stimulates melanin production in the B16F10 cells. In deed, we observed that LY294002 mediated 1.7-fold of increase in melanin production (Fig. 6C).

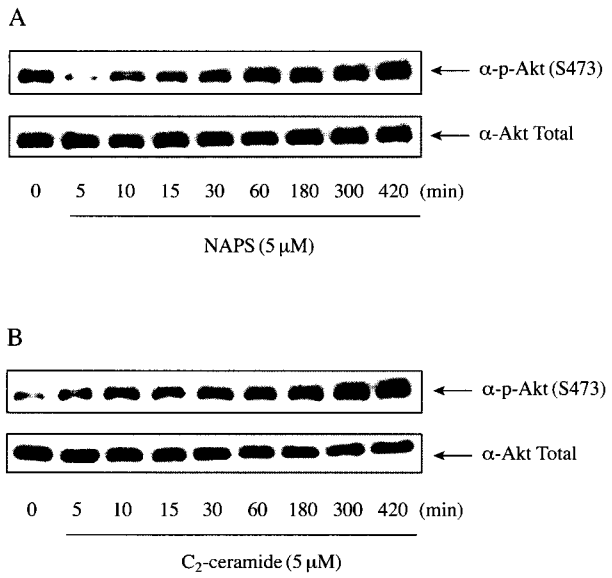

Fig. 5. NAPS (A) and C_2-ceramide (B) induce a time-dependent increase in Akt/PKB-Ser473 phosphorylation in the B16F10 melanoma cells. Cells were incubated in serum-free medium for 12 hours, then treated with 5 μM NAPS (A) or C_2-ceramide (B) for the indicated times. Serum-free cells were used as controls. The protein extracts were harvested, separated via SDS-PAGE, then subjected to Western blot analysis using either total Akt or phospho-Akt/PKB-Ser473 antibodies.

Discussion

A host of previous studies have investigated the diverse biological effects exhibited by ceramide, as sphingolipid metabolites, including ceramide and sphingosine, are well-known to be important in a variety of cellular processes. Several studies have also reported on the anti-proliferative effects of ceramide in human promyelocytic leukemia HL-60 cells[23], as well as in human keratinocytes[24]. With specific regard to melanocytes, C_2-ceramide has been shown to inhibit cell proliferation and melanin synthesis in Malme-3M melanoma cells, a malignant human melanoma cell line[9], and in Mel-Ab cells, immortalized murine melanocytes[10]. Also, both phytosphingosine (PS) and N-acetyl phytosphingosine (NAPS) have been reported to be involved in the heat stress responses of *Saccharomyces cerevisiae*[25], and were shown to induce cell death in Chinese hamster cells[8]. In the present study, we have demonstrated that cell-permeable N-acetyl phytosphingosine (NAPS) can inhibit both tyrosinase activity and melanin production in B16F10 melanoma cells. We also investigated the effects of C_2-ceramide in this regard, and compared the two compounds in terms of their activities.

Both NAPS and C_2-ceramide clearly exerted inhibi-

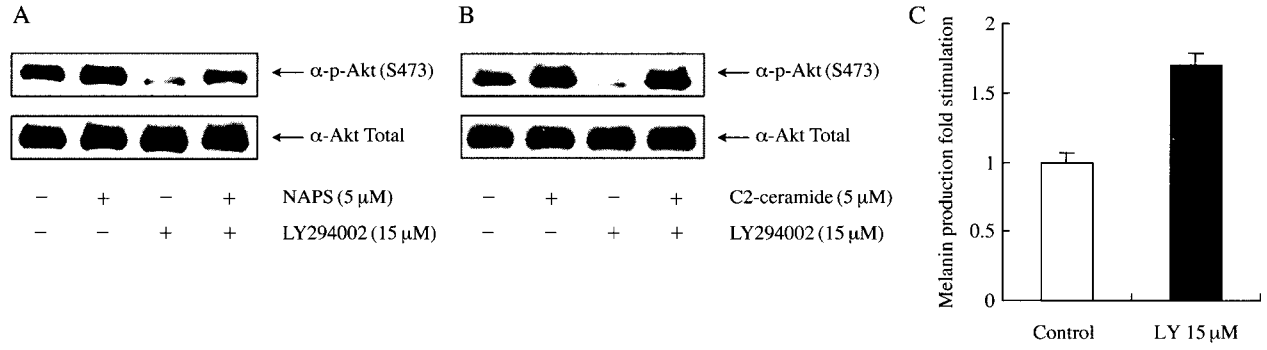

Fig. 6. Effects of pretreatment with LY294002 on the activation of Akt/PKB-Ser473 phosphorylation by NAPS (A) and C_2-ceramide (B). (C) Melanin contents of the cultured B16F10 mouse melanoma cells after treatment with LY294002. In order to characterize Akt/PKB pathway inhibition, cells were grown in serum-free medium for 12 hours, then subjected to 1 hour of pretreatment with 15 μM of LY294002 (LY) prior to 1 hour of stimulation with 5 μM NAPS (A) or C_2-ceramide (B). The control cells were treated with vehicle only for an identical time period. Equivalent amounts of lysate protein (30 μg) were used for Western blotting assays. The cell lysates were separated via SDS-PAGE, and probed for phospho-Akt/PKB-Ser473, then reprobed for total Akt/PKB. (C) In order to determine the melanin contents, the cells were cultured with 15 μM LY294002 for 3 days. Melanin synthesis was clearly induced after the administration of LY294002. Results are expressed as the means of three independent experiments ± S.D.

tory effects on cell growth. At a concentration of 5 μM, NAPS and C_2-ceramide reduced the growth of the B16F10 cells by approximately 20-25%. In order to determine the effects of these compounds with regard to melanogenesis, we conducted an evaluation of tyrosinase activity and melanin content in B16F10 cells which had been treated with NAPS and C_2-ceramide, as tyrosinase is known to be the rate-limiting enzyme in melanin synthesis. According to the results of this study, tyrosinase activity and melanin production both decreased in a dose-dependent manner. When we observed morphological changes occurring in response to treatment with NAPS and C_2-ceramide, we noted that the treated cells appeared bigger and more slender than the control cells (data not sown). The observed reductions in tyrosinase activity and melanin production may also be attributable to the induction of differentiation of B16F10 cells in response to treatment with NAPS and C_2-ceramide. In previous reports, C_2-ceramide was determined to reduce the percentage of cells in S phase, and a concomitant increase in the G0/G1 phase was observed[9,10]. Therefore, both NAPS and C_2-ceramide were expected to regulate the cell cycle in the B16F10 cells, resulting in an inhibition of cell growth and morphological changes. We also evaluated the mRNA expressions of tyrosinase, as well as other melanogenesis-associated proteins, including tyrosinase related protein-1 (TRP-1), and tyrosinase related protein-2 (TRP-2). However, we detected no alterations in the mRNA expression levels of tyrosinase, TRP-1 and TRP-2 in response to treatment with NAPS and C_2-ceramide. Moreover, the expression of tyrosinase protein was also examined via Western blotting methods, and we noted no alterations in the expression of tyrosinase protein. Accordingly, we suggest that tyrosinase activity was modulated by treatment with NAPS and C_2-ceramide, regardless of the protein levels of these enzymes, and we expect that NAPS and C_2-ceramide compete with tyrosine at the active site of tyrosinase. These results compelled us to attempt to gather more evidence regarding the inhibitory activities of NAPS and C_2-ceramide, in order to fully understand the relevant mechanisms. Therefore, we elected to focus on the signaling mechanisms exploited by NAPS and C_2-ceramide in the B16F10 cells.

In both mice and humans, it is now fairly well-established that the pro-opiomelanocortic peptides, adrenocorticotrophic hormone (ACTH) and α-melanocyte stimulating hormone (αMSH), play key roles in the control of pigmentation, and also activate intracellular cAMP and protein kinase A[26]. cAMP increases melanogenesis, primarily via the stimulation of tyrosinase expression. Busca *et al.* demonstrated that cAMP inhibited phosphatidylinositol 3-kinase (PI3-K) in B16 melanoma cells[19]. Additionally, the PI3-K specific inhibitor, LY294002, was shown to stimulate melanogenesis. These observations suggested that the PI3-K pathway might be involved in cAMP-associated melanogenesis. Moreover, melanin contents were shown to be lower in cells that expressed the constitutively active form of the serine-threonine kinase Akt/PKB, which is a prime target of PI3-K. This suggests that signal transduction via Akt is sufficient for the regulation of melanogenesis in human G361 melanoma cells[17]. However, C_2-ceramide has also been reported to inhibit the phosphorylation of Akt, and then induce an inhibition of melanogenesis in both murine and human melanocytes[9,10]. In this study, in order to elucidate the signaling mechanism exploited by NAPS and C_2-ceramide in the B16F10 cells, we investigated the activation of Akt/PKB by NAPS and C_2-ceramide. We detected the activation of Akt/PKB within 5 minutes by C_2-ceramide, and this activation effect persisted for 7 hours. Although Akt/PKB phosphorylation was clearly reduced 5 minutes after the administration of NAPS treatment, it gradually increased in a time-dependent manner. At 1 hour post-treatment, NAPS induced an increase in phosphorylation above control levels, and this effect gradually became more pronounced until 7 hours post-treatment (Fig. 5A). This different signaling pattern may be attributable to the differences in the structures of NAPS and C_2-ceramide. This finding is also not consistent with many previous reports, which demonstrate that C_2-ceramide inhibits Akt phosphorylation. Moreover, LY294002, the specific PI3-K inhibitor used in this study, inhibited not only the induction of Akt/PKB phosphorylation, but also inhibited the reduction of melanin content induced by treatment with NAPS and C_2-ceramide. These results clearly indicate that PI3-K can regulate melanogenesis, and that the activation of Akt is sufficient for melanin synthesis in B16F10 cells. Our results also show that both NAPS and C_2-ceramide inhibit melanogenesis via Akt/PKB activation.

In summary, both NAPS and C_2-ceramide were shown in this study to inhibit B16F10 cell growth, tyrosinase activity, and melanin synthesis, regardless of the levels of tyrosinase, TRP-1, and TRP-2 proteins in the cells. Therefore, we suggest that the depigmenting mechanism of NAPS and C_2-ceramide in the B16F10 cells involves the inhibition of melanosomal tyrosinase activity. We also showed that the Akt/PKB activation associated with NAPS and C_2-ceramide inhibits melanin synthesis in the B16F10 cells. Therefore, NAPS and C_2-ceramide may both regulate

melanogenesis via the activation of Akt/PKB, which is the principal molecule operant in the melanogenesis of B16F10 melanoma cells.

Methods

Materials and Antibodies

The noble phytosphingosine derivative, NAPS, was obtained from Doosan Biotech (Seoul. Korea). C_2-ceramide (N-Acetyl-D-sphingosine semisynthetic, #A7191) was acquired from Sigma (St Louis, MO). The LY294002 was from Calbiochem (San Diego, CA). Synthetic melanin, L-DOPA, mushroom tyrosinase, 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were all purchased from Sigma (St Louis, MO). The phosphorylated Akt/PKB (Ser-473, #P4112), total Akt/PKB (#P1601), and β-actin (#A2228) antibody used in this study were obtained from the Sigma Co. (St Louis, MO). The tyrosinase Ab-1 (#MS-800-P1) was purchased from NeoMarkers (Fremont, CA).

Cell Cultures

The B16F10 cell line (provided by the Korean Cell Line Bank, Seoul, Korea) is a mouse melanoma cell line. The B16F10 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic -antimycotic mixture, at 37°C in a 5% CO_2 atmosphere.

MTT Assay

The cells (1×10^4 cells/well) were seeded in 24-well plates, then incubated with the test substances for 3 days at 37°C in a 5% CO_2 atmosphere. After the addition of 125 μL of MTT solution (2 mg/mL) per well, the plates were incubated for an additional 4 hours. The supernatant was discarded, and the formazan crystal products were dissolved in 1 mL of dimethyl sulfoxide. Optical density was then determined at 570 nm using an ELISA reader (Molecular Devices, Sunnyvale, CA, USA). Cell viability was expressed as a percentage of untreated control cells.

Assay of Tyrosinase Activity

Tyrosinase activity was determined by the method described by Tomita *et al.*[21], with slight modifications. In brief, the cells (1×10^4 cells/well) were plated on 24-well plates. After 1 day, the medium was exchanged for fresh DMEM, containing various concentrations of NAPS or C_2-ceramide, and incubation then continued for an additional 3 days. The cells were then washed in phosphate-buffered saline (PBS) and lysed with 1% Triton X-100/PBS (pH 6.8, 180 μL/well), then frozen for 30 minutes at −80°C. After thawing and mixing, 20 μL of 10 mM L-DOPA was added to each of the wells. The plates were then incubated for an additional 3 hours at 37°C, and the absorbance was measured at 475 nm. The data was standardized using mushroom tyrosinase (>4,000 unit/mg).

Assay of Melanin Contents

The melanin contents in the cultured B16F10 cells were determined using a modified version of the method described by Tsuboi *et al.*[22]. The cells were seeded in 60-mm culture dishes, and the medium was exchanged for fresh DMEM, containing 1-5 μM NAPS or C_2-ceramide. After 3 days, the cells were collected and digested in 1 mL of 1 N NaOH for 30 minutes at 100°C, then centrifuged for 20 minutes at 16,000 × g. The optical density of the supernatant was determined at a wavelength of 405 nm, using an ELASA reader. The absorbance values were then compared to a standard curve obtained with synthetic melanin dissolved and diluted in 1 N NaOH.

Semi-Quantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total cellular RNA was prepared using TRIzol solution (Gibco-BRL, Paisley, UK), in accordance with the manufacturer's instructions. After the preparation of CDNA with oligo d $(T)^{16}$ as a reverse transcriptase primer from the extracted RNA, we conducted PCR amplification. The following oligonucleotide primers were used in the PCR: Tyrosinase sense 5′-TTC AAA GGG GTG GAT GAC CG-3′; antisense 5′-GAC AVC ATA GTA ATG CAT CC-3′: Tyrosinase related protein-1 (TRP-1) sense 5′-GCT GCA GGA GCC TTC TTT CTC-3′; antisense 5′-AAG ACG CTG CAC TGC TGG TCT-3′: Tyrosinase related protein-2 (TRP -2) sense 5′-GGA TGA CCG TGA GCA ATG GCC-3′; antisense 5′-CGG TTG TGA CCA ATG GGT GCC-3′: β-actin sense, 5′-TCA GAA GGA CTC CTA TGT GG-3′; and antisense 5′-TCT CTT TGA TGT CAC GCA CG-3′. The product, approximately 5 μg of total RNA, was then employed for cDNA synthesis. The PCR profile for tyrosinase and β-actin included 35 cycles; 1 minute at 95°C, 1 minute at 55°C, and 1 minute at 72°C. The PCR profile of TRP-1 and TRP-2 included 20 cycles; 1 minute at 94°C, 1 minute at 56°C, and 1 minute at 72°C. The PCR products were then analyzed via electrophoresis on 1.5% agarose gel. Each of the experiments was conducted more than three times.

Western Blotting

The cells were cultured in 60 mm-dishes with the test substances for the times indicated in the Figure Legends. The cells were then collected and lysed in RIPA buffer (containing 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 1 mM PMSF, 1 mM Na_3VO_4, 20 μM okadaic acid, and COMPLETE protease inhibitor cocktail, from Roche). 30 micrograms of protein was loaded onto 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and exposed to the appropriate antibodies. Proteins were visualized using an Amersham Biosciences ECL system, using horseradish peroxidase-conjugated secondary antibodies. The western blot assays were representative of at least three experiments.

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