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Nicotinic Acid Hydroxamate Downregulated the Melanin Synthesis and Tyrosinase Activity through Activating the MEK/ERK and AKT/GSK3β Signaling Pathways

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ABSTRACT: In this study, nicotinic acid hydroxamate (NAH), a nicotinic acid derivative, was found to show dose-dependent inhibition of melanin content and tyrosinase activity of murine melanoma B16F10 cells with or without being cotreated with cAMP stimulators. In the studies on signaling pathways for skin whitening, NAH-treated B16F10 cells resulted in a decrease in the expression of tyrosinase, tyrosinase-related protein-1, and microphthalmia-associated transcription factor (MITF). PD98059 and LY294002 additions were obviously to increase melanin contents in B16F10 cells; however, they were reversed by NAH cotreatments. NAH-mediated increases in the phosphorylation of mitogen-activated protein kinase kinase (MEK)/ERK and AKT/glycogen synthase kinase-3β (GSK3β) were also found, which in turn led to the inhibition of MITF expression and then downregulated tyrosinase and tyrosinase-related protein (TRP)-1 expressions. These results suggest that NAH may be an active component in the inhibition of melanogenesis, which will have potential uses as cosmetics for whitening and need further investigation.

KEYWORDS: *extracellular signal-regulated kinase (ERK), glycogen synthase kinase-3β (GSK3β), nicotinic acid hydroxamate (NAH), melanogenesis, tyrosinase, microphthalmia-associated transcription factor (MITF)*

■ INTRODUCTION

Melanin, present in human melanocytes, plays a crucial role in protecting the skin by absorbing and scattering ultraviolet light from the sun, neutralizing free radicals, and scavenging toxic drugs and chemicals.[1] However, abundant generation of melanin can cause visible hyperpigmentation of the epidermis, which may manifest as melasma, freckles, age spots, or senile lentigines.[2] Tyrosinase (polyphenol oxidase; EC 1.14.18.10), a copper-containing metalloenzyme, is a key enzyme in melanogenic processes and catalyzes the first 2 rate-limiting steps of melanogenesis: hydroxylation of tyrosine to produce dihydroxyphenylalanine (DOPA) and oxidation of DOPA to the corresponding dopaquinone. The dopaquinone is further catalyzed by tyrosinase-related protein-1 (TRP-1) and TRP-2 to produce eumelanin, which has a brown or black color, or nonenzymatically polymerized into pheomelanin, which has a yellow to reddish-brown color.[3]

The microphthalmia-associated transcription factor (MITF) plays an important, physiological role in melanocytes. The MITF is a family of basic helix−loop−helix leucine zipper DNA-binding proteins. The MITF-M, one of the transcription factors specifically expressed in melanocytes and melanoma cells, binds to the tyrosinase promoter to form a DNA−protein complex, and then to transactivate tyrosinase and TRP-1 during melanogenesis.[4] The cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) signaling pathway plays a key messenger to increase MITF expressions at the mRNA and protein levels.[5] Intracellular cAMP levels are regulated by several cAMP-elevating agents, such as α-melanocyte-stimulating hormone (α-MSH), isobutylmethylxanthine (IBMX), and forskolin.[6,7] On the other hand, mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinases (ERK) and phosphatidylinositol 3-kinase(PI3K)/AKT/glycogen synthase kinase-3β (GSK3β) signaling pathways also participate in regulating melanogenesis by enhancing MITF degradation and inhibition of MITF binding to tyrosinase promoter site, respectively.[8,9]

Nicotinamide (niacinamide, 3-pyridine carboxamide), which is a component of two important coenzymes, NADH and NADPH, is a derivative of nicotinic acid obtained through synthesis in the body or from dietary sources such as meat, fish, milk, egg, and nuts in which the amide moiety ($-CONH_2$) is bound to the C-3 of the pyridine ring; while nicotinic acid hydroxamate (NAH), also known as 3-pyridine carbohydroxamic acid, is one of the nicotinic acid derivatives in which the hydroxamic acid moiety (−CONHOH) is bound to the C-3 of the pyridine ring. NAH is a urease inhibitor and has low toxicity in animals.[10] Moreover, our recent study showed that NAH was a potent monophenolase and diphenolase inhibitor of mush-

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room tyrosinase in vitro, and the related structures of methyl nicotinate and nicotinamide showed less inhibition against mushroom tyrosinase.[11] In addition, horseradish peroxidase and mushroom tyrosinase were inhibited by *m*-chlorobenzohydroxamic acid, salicylhydroxamic acid, and benzohydroxamic acid.[12] Rho et al.[13] reported that different synthetic hydroxamic acid derivatives exhibited antityrosinase activities; however, the real mechanisms are still unknown. In fact, the tyrosinase inhibitory activities in enzymatic reaction were not equal to the reduced melanin levels in cell models. Therefore, we used cell models to study the effects of NAH on antimelanogenesis in B16F10 melanoma cells and to propose the possible antipigmental mechanisms.

MATERIALS AND METHODS

Materials. L-DOPA, forskolin, IBMX, LY294002, α-MSH, NAH, PD98059, sulforhodamine B (SRB), trichloroacetic acid, and 0.4% trypan blue were all purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti-β-actin antibody, horseradish peroxidase-conjugated goat anti-mouse, and horseradish peroxidase-conjugated goat anti-rabbit IgG were from Sigma Chemical Co. (St. Louis, MO, USA). Anti-MITF antibody was from Abcam Inc. (Cambridge, MA, USA). Antibodies against MEK, phospho-MEK, ERK1/2, phosphor-ERK1/2 and AKT were from Cell Signaling Technology, Inc. (Beverly, MA, USA). Antibodies against tyrosinase, TRP-1, TRP-2, and p-AKT (Ser473) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). GSK-3β and phosphor-GSK-3β (Ser9) antibodies were from GeneTex, Inc. (San Antonio, TX).

Cell Culture. Murine melanoma B16F10 cells (BCRC 60031) were purchased from the Bioresource Collection and Research Center (BCRC), Food Industry Research and Development Institute (Hsinchu, Taiwan), and cultured in Dulbecco's modified Eagle medium (DMEM), 10% fetal bovine serum (FBS), and penicillin/streptomycin solution (10,000 units/mL of penicillin and 10 mg/mL of streptomycin). The cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

Cell Viability Assay. The SRB dyes were used for cell viability assays. Briefly, the cells were seeded onto a 24-well microtiter plate, and various concentrations of NAH were added. Then, the cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2 for 3 days, fixed by 10% trichloroacetic acid at 4 °C for 1 h, washed with distilled water, and then stained with 0.4% SRB (dissolved in 1% acetic acid) for 30 min. After being washed 4 times with 1% acetic acid to remove the unbound dye in each well, air-dried, and then bound, dyes were dissolved in 500 μL of 10 mM Tris-HCl buffer (pH 7.9). The blank group used medium only, and the control group used deionized water instead of NAH. The absorbance was measured at 540 nm by an ELISA reader (Sunrise, TECAN, Männedorf, Switzerland) and was expressed relative to the control (%).

Determination of Cellular Tyrosinase Activity. After incubation with different concentrations of NAH for 3 days, the B16F10 cells were harvested and then lysed by cold RIPA buffer containing a protease inhibitor cocktail (P-8340, Sigma Chemical Co.). Further, the protein contents in cell homogenates were quantified using the BCA Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL, USA). The reaction mixture (200 μL) contained 2.5 mM L-DOPA, 250 mM phosphate buffer (pH 6.8), and cell homogenates (100 μg of protein of each treatment). After incubation at 37 °C for 1 h, the absorbance of the solution was measured at 475 nm and was expressed relative to the control (%). The blank group used medium only, and the control group used deionized water instead of NAH.

Determination of Melanin Contents. Different concentrations of NAH were added to the B16F10 cells, and the cells were incubated for 3 days. The blank group used medium only, and the control group used deionized water instead of NAH. The melanin contents were expressed relative to the control (%). On the other hand, the NAH-treated cells were centrifuged, washed with 10 mM Tris buffer (pH 7.9), and then photographed for quality of melanin formation. In addition, B16F10 melanoma cells were either pretreated with 100 nM α-MSH and then treated with different concentrations of NAH for 3 days or cotreated with 1 μM forskolin or 100 μM IBMX with different concentrations of NAH for 3 days. The melanin contents of untreated cells were considered as 100% in cAMP elevator additions. After removal of the medium, the cultured cells were centrifuged, saved, washed twice with PBS, and then lysed with 1 N NaOH at 90 °C for 30 min. The absorbance of the extracted melanin was measured at 405 nm.

Western Blot Analysis. For investigating the signaling pathway, B16F10 melanoma cells were treated with NAH (5, 15, 25, and 50 μM) and then lysed, and the supernatants were saved and subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). After electrophoresis, the gels were equilibrated with Tris-glycine buffer (pH 8.3) and transferred onto immobile polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford, MA). The PVDF membranes were blocked with 1% gelatin in NaCl/EDTA/Tris (NET) solution for 1 h at room temperature and incubated overnight at 4 °C with each of the primary antibodies. The β-actin, MITF, ERK1/2, phospho-ERK1/2, MEK, phospho-MEK, AKT, GSK-3β, and phospho-GSK-3β antibodies were used each in a 1000-fold dilution; tyrosinase, TRP-1, TRP-2, and phospho-AKT antibodies were used each in 500-fold dilutions (in 0.25% gelatin in NET solution). The PVDF membranes were washed thrice with phosphate buffered saline Tween-20 (PBST) for 10 min. Thereafter, horseradish peroxidase-conjugated IgG (goat anti-mouse or goat anti-rabbit IgG) solution (1000-fold dilution in 0.25% gelatin in NET solution) was added, and the membrane was washed again using 1× PBST. The immunoblots were visualized using 3-amino-9-ethylcarbazole and hydrogen peroxide solution. The β-actin was used as an internal control. The inhibitory effects of NAH treatment on protein expression were quantified and expressed as the relative density (% area) by using the Syngene G:bBOX imaging system (Syngene, Cambridge, U.K.) equipped with the GeneSnap software (Syngene), and the relative density in the absence of NAH treatment was considered as 100%.

Statistical Analyses. All data were calculated as mean ± SD and were expressed as a percentage of the average values of the control group. Multiple group comparisons were performed using one-way ANOVA followed by post hoc Tukey's test. Differences in the two groups were considered statistically significant at $P < 0.05$. Statistical analysis was performed using the GraphPad Prism 5.0 software (San Diego, CA, USA).

RESULTS

NAH-Induced Inhibition of Hyperpigmentation in B16F10 Cells. B16F10 cells were treated with NAH (5, 15, 25, and 50 μM) for 3 days and then for the cell viability assays. NAH showed no apparent cytotoxicity to B16F10 cells under tested concentrations (Figure 1A). However, dose-dependent reductions in the melanin content and tyrosinase activity of B16F10 cells (Figure 1B) were found under NAH treatments and showed significant difference ($P < 0.001$, ***) compared to the blank. The photographs of cell pellets also showed clear reductions of pigments in NAH-treated B16F10 cells (Figure 1C). These results showed that NAH exhibited antipigmental effects in B16F10 cells.

Effects of NAH on Melanin Content and Tyrosinase Activity Induced by cAMP Elevators. To investigate effects of NAH on cAMP elevator-induced hyperpigmentation, B16F10 cells were treated with α-MSH, IBMX, and forskolin in the presence of NAH (25 and 50 μM) for 72 h, and the cell melanin contents and tyrosinase activities were determined and are shown in Figure 2. The cAMP elevators showed significant difference ($P < 0.001$, #) and increase in tyrosinase activities (Figure 2A), and melanin contents (Figure 2B), of B16F10 cells compared to untreated blank; however, cotreatments of cAMP elevators with NAH showed significant difference ($P <$

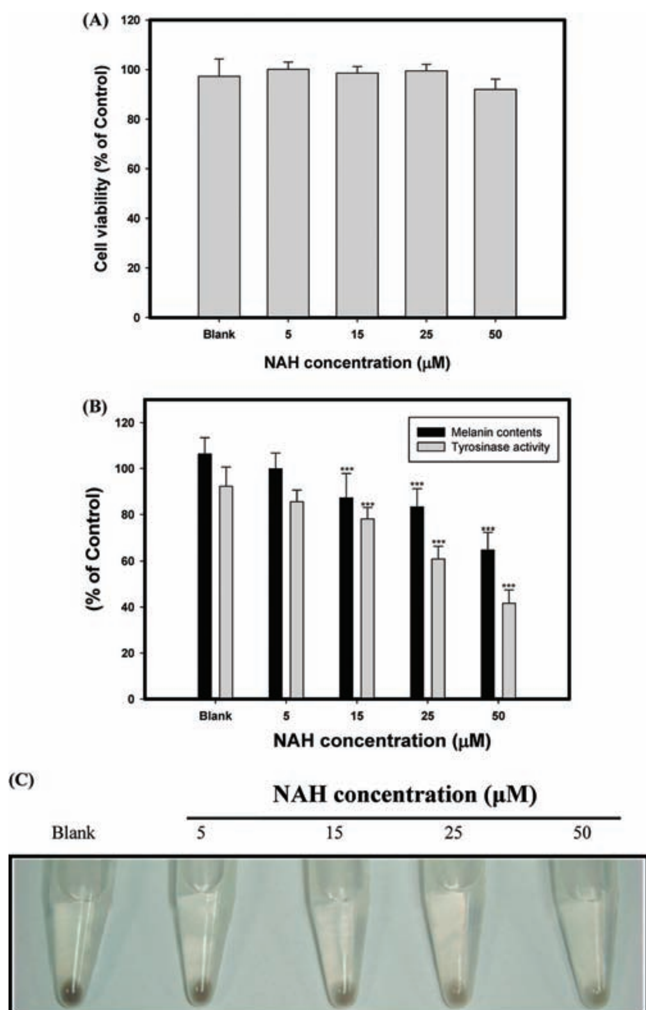

Figure 1. Effects of different concentrations (5, 15, 25, and 50 μM) of NAH on melanogenesis of B16F10 melanoma cells. Cell were treated with NAH for 72 h and then for (A) cell viability assay and (B) melanin contents and tyrosinase activity determination. (C) After being cultured for 72 h, the cell pellets were collected and then photographed for comparisons. Data were expressed as a percentage of control of three independent experiments. $P < 0.001$ (***), each treatment compared to blank.

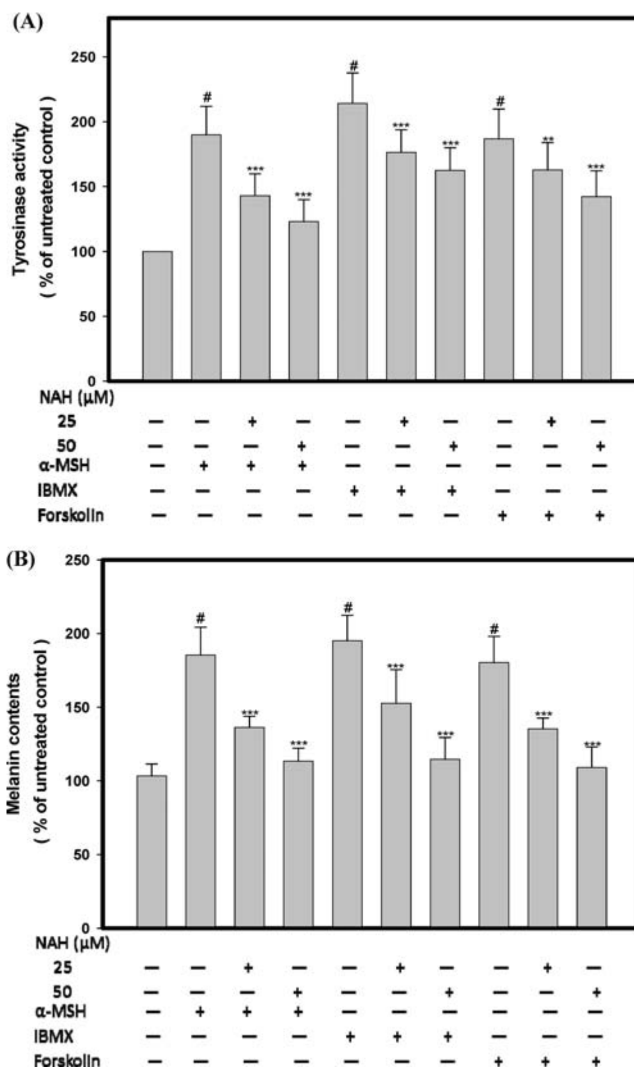

Figure 2. Effects of NAH (25 and 50 μM) on melanogensis of B16F10 melanoma cells in the presence of melanogenic inducer, including α-MSH (100 nM), IBMX (100 μM), and forskolin (1 μM) for (A) tyrosinase activity assay and (B) melanin contents. Data were mean ± SD expressed as a percentage of untreated blank (the left column) of three independent experiments. $P < 0.001$ (#), each inducer treatment compared to untreated blank (the left column); $P < 0.01$ (**) and $P < 0.001$ (***), each inducer cotreated with NAH compared to inducer-treated one.

0.01, **; $P < 0.001$, ***) and decrease, in a dose-dependent matter, of tyrosinase activities (Figure 2A), and melanin contents (Figure 2B), of B16F10 cells compared to inducer-treated cells. The melanin contents, but not tyrosinase activity, in treated cells of each cAMP elevator in the presence of 50 μM NAH were shown to be close to that of the untreated blank (Figure 2B). These results showed that NAH could retard melanogenesis in the presence of cAMP elevators in B16F10 melanoma cells.

NAH Downregulated Tyrosinase, TRP-1, and MITF Expressions through the Activation of MEK/ERK and AKT/GSK3β Signaling Pathway in B16F10 Cells. The effects of NAH on TRP-1, TRP-2, and MITF expressions were investigated using the Western blot technique (Figure 3A), and the intensities were quantified using a densitometer equipped with the GeneSnap software (Syngene) (Figure 3B). The results revealed that NAH showed significant difference compared to the blank ($P < 0.05$, *; $P < 0.01$, **; $P < 0.0001$, ***) and to reduce protein expressions, including tyrosinase, TRP-1, and MITF, but not for TRP-2. It was proposed that NAH might downregulate MITF expression, which in turn might inhibit the expressions of tyrosinase and TRP-1, and finally resulted in the retardation of melanogenesis. Previous studies revealed that MEK/ERK and PI3K/AKT/GSK3β signaling pathways participate in regulating melanogenesis.[8,9] Therefore, the upstream signal pathways underlying the antipigmental effects of NAH were investigated, including ERK, MEK, AKT, and GSK-3β and their phosphorylated patterns, by Western blot analysis (Figure 4A). It was clear that NAH could increase the levels of phosphorylated MEK (p-MEK), which in turn would increase the levels of phosphorylated ERK (p-ERK1/2, lowered band, arrow indicated). In addition, both phosphorylated AKT (p-AKT) and phosphorylated GSK-3β (p-GSK-3β) were also increased after NAH treatments. Therefore, the specific inhibitors of PD98050 and LY294002 in the presence of NAH for melanin

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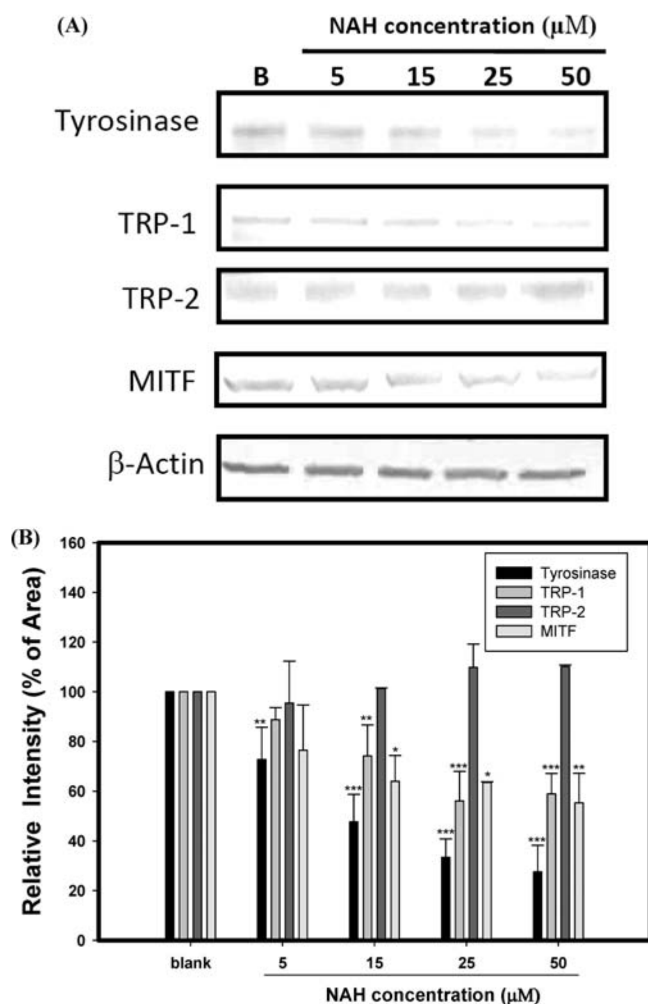

Figure 3. Inhibition of TRP-1, tyrosinase, and MITF protein expressions by different concentrations (5, 15, 25, and 50 μM) of NAH treatments. (A) Changes of melanogenic enzymes (tyrosinase, TRP-1, and TRP-2), and MITF in B16F10 melanoma cells by Western blotting, and (B) the protein expressions were quantified by the scanning densitometer using β-actin as an internal standard and expressed as a percentage of relative intensity (% area of blank) in three independent experiments. $P < 0.05$ (*), $P < 0.01$ (**), or $P < 0.0001$ (***), NAH treatment was compared to the blank of each protein expression.

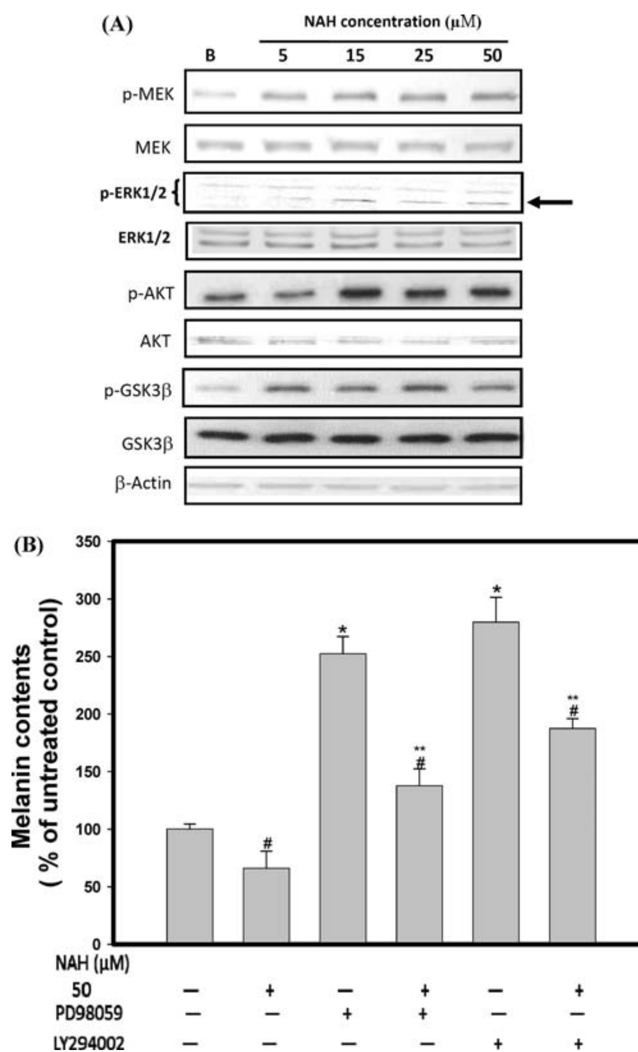

Figure 4. Effects of different concentrations (5, 15, 25, and 50 μM) of NAH on downregulation of the phosphorylated ERK and PI3K in B16F10 melanoma cells. (A) Changes of signal-related ERK, MEK, AKT, GSK-3β, and each phosphorylated protein expression by Western blotting using β-actin as an internal standard. (B) Changes of melanin contents which were pretreated with PD98059 or LY294002 (20 μM) for 1 h and then cultured with or without 50 μM NAH for 72 h. $P < 0.001$ (*), PD98059 and LY294002 treatment compared to untreated blank (the left column); $P < 0.001$ (#), NAH treatment or NAH cotreated with each inhibitor compared to each inhibitor-treated one; $P < 0.001$ (**), NAH cotreated with each inhibitor compared to NAH-treated only.

formations were investigated, and the results are shown in Figure 4B. The B16F10 cells were pretreated in the presence or absence of PD98059 or LY294002 for 1 h, and then treated with 50 μM NAH for 72 h, and the melanin contents were determined. It was found that melanin levels were 2.5-fold increased in each inhibitor-treated cell and showed significant difference ($P < 0.001$, *) compared to untreated blank. However, each inhibitor in the presence of 50 μM NAH showed significant reductions in the melanin levels ($P < 0.001$, #) compared to each inhibitor-treated one. It was noted that each inhibitor pretreatment and then NAH treatment showed significantly higher melanin contents ($P < 0.001$, **) compared to NAH-treated only. These results suggested that NAH-induced hypopigmentation might connect closely with the MEK/ERK and PI3K/AKT pathways. These results suggested that NAH might induce phosphorylations of MEK, ERK, AKT, and GSK3β which were closely associated with the function and fate of MITF.

■ DISCUSSION

Hydroxamic acid derivatives have many biological activities such as inhibition of metalloenzymes and antimalarial, antibacterial, fungicidal, antimetastatic, antitumor, and anti-inflammatory activities.[14,15] Our recent results showed that NAH was a potential mushroom tyrosinase inhibitor in vitro, for which the concentrations of half-inhibition (IC_{50}) against monophenolase and diphenolase activities were 2 μM and 1 μM, respectively, compared to kojic acid of 62 μM and 180 μM, respectively.[11] In this study, under non-cytotoxic concentrations (below 50 μM), NAH treatment could dose-dependently inhibit tyrosinase activity and reduce the melanin contents in B16F10 melanoma cells. Moreover, kojic acid below 0.3 mM did not show reduced melanin contents of B16F10 melanoma

cells in the same conditions (data not show). It was suggested that NAH might be a potent compound for hyperpigmentation treatment.

Some cAMP elevators, such as α-MSH, IBMX, and forskolin, could increase melanogenesis by different mechanisms.[16] The α-MSH could increase the intercellular cAMP concentrations by binding to the melanocortin 1 receptor, while IBMX and forskolin were shown to increase the intercellular cAMP concentrations, respectively, by means of cAMP phosphodiesterase inhibition and directly activating adenylyl cyclase.[17] Our present results showed that each cAMP elevator could induce hyperpigmentation in B16F10 cells, however, NAH treatment in the presence of each cAMP elevator could apparently induce hypopigmentation by decreasing the tyrosinase activities and melanin levels in B16F10 cells, especially for the latter in which the treated cells of each cAMP elevator in the presence of 50 μM NAH showed results close to that of the untreated blank (Figure 2B). However, cAMP concentrations in B16F10 melanoma cells were not significantly changed after NAH treatment (data not shown), therefore, it was suggested that the antimelanogenic effects of NAH might participate in signal pathway regulation other than direct tyrosinase inhibition as our previous report.[11] Therefore, the expressions of melanogenic enzymes and MITF were checked after NAH treatments.

In mammalian cells, MITF is the main transcriptional factor involved in the regulation of melanogenic proteins, such as tyrosinase and TRP-1.[4] The present result revealed that NAH showed significant difference compared to the blank and to reduce protein expressions, including tyrosinase, TRP-1, and MITF, but not for TRP-2 (Figure 3). It was possible that NAH might upregulate MITF degradation to participate in antipigmentation. Transient expression assays showed that MITF-M had no capacity to transactivate the TRP-2 promoter, therefore, the TRP-2 is expressed before tyrosinase and TRP-1 during embryogenesis,[18] and it was suggested that, during melanogenesis, the mechanisms associated with TRP-2 expression might be different from those underlying TRP-1 expression.

In melanocytes and melanoma cells, elevation of the intracellular cAMP content resulted in the activation of the Ras/ERK cascade in which the activation of Ras/ERK could phosphorylate MITF on Ser73 and promote its degradation, thereby leading to an inhibition of tyrosinase expression and melanogenesis, and specific MEK inhibitor of PD98059 could finally increase tyrosinase expression and activity.[19] On the other hand, PI3K phosphorylated AKT, after which the activated AKT then phosphorylated GSK3β on Ser 9 and promoted GSK3β inactivation, while the unphosphorylated GSK3β could phosphorylate MITF on Ser 298, which in turn increased the binding to the MITF-M box of the tyrosinase promoter, leading to melanogenesis.[20] Therefore, the elevated intracellular cAMP also induced a strong inhibition of AKT phosphorylation, and LY294002, a specific pharmacological inhibitor of PI3K, stimulated melanogenesis which associated with AKT/GSK3β signaling pathways. Treatment of B16F10 cells with PD98059 and LY294002 drastically increased the melanin contents in B16F10 cells, however, cotreatment with NAH decreased the melanin contents in these cells (Figure 4). These results suggested that the hypopigmentation of NAH might participate in regulating the MEK/ERK and AKT/GSK3β signaling pathways. In the present study, it was found that NAH was shown not only to increase levels of phosphorylated MEK and phosphorylated ERK but also to increase levels of phosphorylated AKT and phosphorylated GSK-3β (Figure 4B), in a dose-dependent manner. Previous reports have indicated that some compounds, such as hinokitiol, methyl-β-cyclodextrin, and sphingosine-1-phosphate, decrease melanogenesis via modulation of the ERK pathway.[21,22] Notably, haginin A showed antimelanogenesis by tyrosinase inhibitory activity and downregulated the ERK and PI3K pathways.[23] Moreover, MITF was targeted for degradation via the hyperactivated ERK pathway.[24] Hyperactivated ERK mediated the formation of dual phosphorylated MITF molecules, which showed altered ubiquitination and subsequent degradation, and this leads to reduced melanogenic levels.[25] Furthermore, AKT and GSK-3β were downstream molecules of the PI3K-mediated signaling pathway and were also involved in the melanogenesis of G361 and B16 melanoma cells.[9,26] Therefore, it was proposed that the antipigmentation of NAH might activate the MEK/ERK signal pathway to increase MITF degradation which resulted in the reduced protein MITF expressions and also activate the AKT/GSK3β signaling pathway to reduce MITF phosphorylation on Ser298 which blocked the binding to the MITF-M box of the tyrosinase promoter and then downregulated tyrosinase and TRP-1 expressions.

In conclusion, our present results showed that NAH-induced phosphorylation of MEK/ERK and AKT/GSK-3β might contribute to the hypopigmentation effect. The hydroquinone, kojic acid, and arbutin were popular antipigmentation agents and were widely used in the cosmetic industry. Some studies have raised concerns of adverse effects and carcinogenic potential of some common whitening agents.[27] Therefore, current research on cosmetic products has been focused on identifying alternative sources of safe whitening agents. Sugihara and Tastsumi[28] reported that liver aldehyde oxidase could metabolize NAH to nicotinamide, which may have low toxicity in animals. In addition, a clinical trial involving oral administration of NAH in animal models showed that NAH could reduce blood ammonia levels and then improve neuropsychiatric symptoms in humans and animals with hyperammonemia.[29,30] These results suggested that NAH might be an active compound in the inhibition of melanogenesis which will have potential uses as cosmetics for whitening and need further investigation.

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Notes

The authors declare the following competing financial interest(s): Taiwan (098127575) and US (12/609,718) patents pending.

ABBREVIATIONS USED

ERK, extracellular signal-regulated kinase; GSK3β, glycogen synthase kinase-3β; NAH, nicotinic acid hydroxamate; MAPK, mitogen-activated protein kinase; MITF, microphthalmia-associated transcription factor; PI3K, phosphatidylinositol 3-kinase; SRB, sulforhodamine B; TRP, tyrosinase-related protein; L-DOPA, 3,4-dihydroxy-L-phenylalanine.

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