Salicylamide Enhances Melanin Synthesis in B16F1 Melanoma Cells

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Abstract

Salicylamide, a non-steroidal anti-inflammatory drug (NSAID), is used as an analgesic and antipyretic agent. We have previously shown that several NSAIDs have anti-melanogenic properties in B16F1 melanoma cells. In contrast, we have found that salicylamide enhances melanin contents in B16F1 melanoma cells; however, the underlying mechanism is not known. Therefore, we investigated the mechanism through which salicylamide stimulates melanogenesis. Interestingly, salicylamide enhanced diphenolase activity in a cell-free assay. Western blotting and real-time RT-PCR revealed that salicylamide increased tyrosinase expression via transcriptional activation of the *Mitf* gene. Together, our results indicate that salicylamide could be used as an anti-hypopigmentation agent for skin and/or hair.

Key Words: Salicylamide, Melanogenesis, Tyrosinase, Melanoma, Mitf

INTRODUCTION

Non-steroidal anti-inflammatory drugs (NSAIDs) can directly suppress cyclooxygenase (COX) activity. COX catalyzes the conversion of arachidonic acid to prostaglandins (Weissmann, 1991; Desborough and Keeling, 2017). Therefore, NSAIDs can reduce pain, decrease fever, and prevent inflammation. Moreover, many studies have demonstrated that NSAIDs can prevent the development of cancer in several tissues and can induce cell death and/or cell proliferation arrest in cancer cell lines (Piazuelo and Lanas, 2015; Yiannakopoulou, 2015; Seetha *et al*., 2020; Shi *et al*., 2020; Zappavigna *et al*., 2020). Acetylsalicylic acid is one of the most commonly used and well-studied drugs globally. It not only exert the typical effects of NSAIDs but also inhibits platelet aggregation (Michalska-Malecka *et al*., 2016). Therefore, acetylsalicylic acid is effective for prevention of heart attacks, stroke, and cerebral thrombosis (Weissmann, 1991; Butenas *et al*., 2001).

Melanin is a key determinant of skin color, and melanin content is increased by exposure to UV radiation (Korner and Pawelek, 1982). Melanin has an important role in the absorption of harmful UV radiation and in the reduction of UV-induced DNA damage. Tyrosinase is a key enzyme in melanogenesis that catalyzes two different chemical reactions: hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA) and oxidation of DOPA to DOPAquinone (Decher *et al*., 2006; Matoba *et al*., 2006). DOPAquinone is converted to DOPAchrome and then to 5,6-dihydroxyindole (DHI) or indole 5,6-quione 2-carboxylic acid (DHICA). There are two other enzymes involved in melanin synthesis: TRP-2 catalyzes the conversion of DOPAchrome to DHICA, and TRP-1 catalyzes the oxidation of DHICA (Jackson *et al*., 1992; Tsukamoito *et al*., 1992; Kameyama *et al*., 1993). Microphthalmia-associated transcription factor (Mitf) plays an important role in melanin synthesis and can activate the melanogenic gene transcription of *tyrosinase*, *TRP-1* and *TRP-2* (Bertolotto *et al*., 1998; Steingrimsson *et al*., 2004).

Recently, we found that acetylsalicylic acid can also inhibit melanin synthesis in murine melanoma cells (Sato *et al*., 2008). We demonstrated that several other NSAIDs could downregulated the expression of melanogenic genes, including *Mitf* and tyrosinase (Sato *et al*., 2011). Salicylamide is an over-the-counter drug used as an analgesic and antipyretic agent. Recently, Alhashimi *et al*. (2019) reported that salicyl-

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amide exerted antibacterial effects in multidrug-resistant gonorrhea. Moreover, Chowdhury *et al*. (2021) suggested that salicylamide is a promising drugs for the treatment of the novel coronavirus disease 2019 (COVID-19). The identification of new NSAIDs effects is very important for the discovery of additional opportunities for drug repositioning. To the best of our knowledge, there has been no study on the effect of salicylamide on melanin synthesis. In this study, we investigated the effect of salicylamide on melanogenesis in B16F1 melanoma cells and elucidated the underlying mechanism.

MATERIALS AND METHODS

Cell culture

B16F1 melanoma cells (provided by the Riken BioResource Center, Ibaraki, Japan) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS), 50 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified atmosphere containing 5% CO_2.

Cell viability and proliferation

The effect of salicylamide on cell viability and proliferation was assessed by the trypan blue exclusion test. Briefly, B16F1 melanoma cells were treated with or without salicylamide for 3 days at the indicated time points. After treatment, the cells were rinsed once with phosphate-buffered saline (PBS) and resuspended in trypsin/EDTA (Life Technologies, Carlsbad, CA, USA), and the number of viable and dead cells was determined using a Fuchs-Rosenthal cytometer under a light microscope. The percentage of viable cells was calculated as follows;

Number of viable cells/Total number of cells×100.

To determine the cell proliferation rate, the total number of viable cells was counted and compared to an untreated control.

Flow cytometry for cell cycle analysis

To assess the effect of salicylamide on the cell cycle, we performed flow cytometric analysis. First, B16F1 melanoma cells were seeded in a 60 mm dish and incubated for 24 h. After salicylamide treatment for 3 days, the cells were collected and centrifuged at 1,000 rpm for 10 min. Subsequently, the cells were washed with PBS and fixed with ice-cold 80% ethanol. The samples were stored at –20°C for at least 24 h. After permeabilization and fixation, ethanol was removed. Next, the cells were washed twice with PBS, treated with RNase A for 30 min at 37°C, and stained with propidium iodide (PI; Sigma) at 50 μg/mL. Before the analysis, the cells were filtered through a nylon mesh to remove cell debris. The fluorescence intensity of PI was measured using a BD FACS caliber (BD Biosciences, Franklin Lakes, NJ, USA).

Mushroom tyrosinase activity

The direct effect of salicylamide on monophenolase and diphenolase activities was determined with a cell-free assay system using mushroom tyrosinase (Sato and Toriyama, 2011; Nishio *et al*., 2016). In the monophenolase assay, L-tyrosine was used as substrate. First, 80 μL of 0.1 M phosphate buffer (pH 6.8), 80 μL of 1.25 mM L-tyrosine and 20 μL of DMSO with or without salicylamide were mixed. After adding

20 μL of 500 units/mL mushroom tyrosinase (Sigma) aqueous solution, the reaction was performed at 25°C. Enzyme activity was monitored every 30 seconds for 6.0 min at 475 nm using a microplate reader (SH-9000 Lab, Hitachi High-Tech Science Co., Tokyo, Japan). The percentage of monophenolase activity was calculated as follows:

100–[(A–B)/A×100],

where A represents the difference in the absorbance of the control sample between an incubation time of 3.0 and 6.0 min, and B represents the difference in the absorbance of the test sample over the same incubation period.

The diphenolase activity assay was performed using L-3,4-dihydroxyphenylalanine (L-DOPA, Sigma) as the substrate. First, 300 μL of 0.5 mg/mL L-DOPA solution, 50 μL of salicylamide in DMSO, and 1.1 mL of 0.1 M phosphate buffer (pH 6.8) were mixed. The mixture was preincubated at 25°C for 10 min before the addition of 50 μL of 1,000 units/mL mushroom tyrosinase (Sigma) in aqueous solution, and the reaction was monitored at 475 nm. The percentage of tyrosinase activity was calculated as follows:

100–[(A–B)/A×100],

where A represents the difference in the absorbance of the control sample between an incubation time of 0.5 and 1.0 min, and B represents the difference in the absorbance of the test sample over the same incubation period.

Measurement of tyrosinase activity in cell extract

To obtain intracellular tyrosinase, B16F1 melanoma cells were seeded on 90 mm dishes. After 24 h of incubation, the cells were treated with α-MSH (10 nM) for 3 days and then lysed with RIPA lysis buffer containing a protease inhibitor cocktail. After freezing and thawing, the cell lysates were centrifuged at 15,000 rpm for 5 min at 4°C, and the supernatants were collected. The protein concentrations in the lysate were evaluated using XL-Bradford protein assay kit (Integrale, Tokyo, Japan). Then, 80 μL of 0.1 M phosphate buffer (pH 6.8), 80 μL of 0.5 mg/mL L-DOPA in 0.1 M phosphate buffer, 20 μL of sample in DMSO, and 20 μL of cell lysate were placed in a 96-well plate and mixed well. The absorbance was measured at 475 nm using a microplate reader for 2 hours at 37°C. The percentage of tyrosinase activity was calculated as follows:

100–[(A–B)/A×100],

where A represents the difference in the absorbance of the control sample between an incubation time of 60 and 120 min, and B represents the difference in the absorbance of the test sample over the same incubation period.

Melanin content

Melanin content was measured as previously described (Nishio *et al*., 2016). B16F1 melanoma cells were treated with or without salicylamide (250-1,000 μM) for 3 days. After treatment, the cells were collected by trypsinization and counted using a cytometer. Subsequently, the cells were washed and lysed by boiling in 2 M NaOH. Melanin content was spectrophotometrically analyzed at 405 nm absorbance and normalized using synthetic melanin (Sigma).

DOPA staining in gels

The DOPA staining was carried out as described previously (Nishio *et al*., 2016). B16F1 melanoma cells were treated with or without salicylamide (250-1,000 μM) for 3 days. After treatment, the cells were collected by trypsinization and lysed in

0.1 M sodium phosphate buffer (pH 6.8) containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/mL aprotinin, and 10 μg/mL leupeptin (but without mercaptoethanol or heating). After electrophoresis, SDS-PAGE gels were stained with 0.5 mg/mL L-DOPA for 2 h at 37°C. Gel images were collected with WSE-6100 LuminoGraph 1 (ATTO Corporation, Tokyo, Japan), and densitometric analysis was carried out using CS analyzer software (ATTO Corporation).

Western blotting

To elucidate protein levels, western blotting analyses were performed. Treated cells were lysed using a RIPA lysis buffer system (Santa Cruz Biotechnology, TX, USA). Whole cell lysates were separated by SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Merck Millipore, Burlington, MA, USA). After blocking with 5% skimmed milk in PBS containing 0.1% Tween 20, the membranes were probed overnight with specific primary antibodies at 4°C and were further incubated with horseradish peroxidase-conjugated secondary antibodies. Bound antibodies were detected by chemiluminescence using an ImmunoStar zeta (Fujifilm Wako Pure Chemical, Osaka, Japan) following the manufacturer's instructions. Antibodies to tyrosinase, TRP-1, TRP-2, Mitf, β-actin, anti-rabbit IgG, anti-goat IgG, and anti-mouse IgG were purchased from Santa Cruz Biotechnology.

Quantitative RT-PCR

To investigate the effects of salicylamide on melanogenic gene expression, quantitative RT-PCR analysis was performed. B16F1 melanoma cells were treated with or without salicylamide for 3 day. Total RNA was extracted from salicylamide-treated cells usig ISOGEN 2 (Nippongene, Tokyo, Japan). Reverse-transcription was performed utilizing the TaKaRa RT-PCR kit (TaKaRa, Kyoto, Japan).

Primers used for quantitative PCR were as follows: tyrosinase upstream 5'-TTG CCA CTT CAT GTC ATC ATA GAA TAT T-3', downstream 5'-TTT ATC AAA GGT GTG ACT GCT ATA CAA AT-3'; TRP-1 upstream 5'-ATG CGG TCT TTG ACG AAT GG-3', downstream 5'-CGT TTT CCA ACG GGA AGG T-3'; TRP-2 upstream 5'-CTC AGA GCT CGG GCT CAG TT-3', downstream 5'-TGT TCA GCA CGC CAT CCA-3'; Mitf upstream 5'-CGC CTG ATC TGG TGA ATC G-3', downstream 5'-CCT GGC TGC AGT TCT CAA GAA-3'; and GAPDH upstream 5'-CGT CCC GTA GAC AAA ATG GT-3', downstream

5'-TTG ATG GCA ACA ATC TCC AC-3'. PCR amplification was conducted using a 7500 fast real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA), THUNDERBIRD SYBR qPCR mix (TOYOBO, Osaka, Japan), synthesized cDNA, primers, and distilled water according to the manufacturer's instructions. The real-time PCR cycle conditions were as follows: 1 cycle at 95°C for 2 min, followed by 40 cycle at 95°C for 5 s and 60°C for 30 s. All samples were run in triplicates, and all data are expressed relative to the corresponding calculated GAPDH threshold cycle (ΔCt) values. The calibrated ΔCt values (ΔΔCt) for each sample and for the internal control (GAPDH) were calculated according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), ΔΔCt=ΔCt treated sample–ΔCt control sample.

Results are expressed as the relative mRNA levels compared to controls.

Statistical analysis

Data are expressed as mean ± standard deviation (SD). The significance of differences between experimental conditions was determined using Dunnett's test.

RESULTS

Salicylamide did not induce cell death but suppressed cell proliferation

First, we performed the trypan blue exclusion test to assess cell viability. In this assay, cells were treated with salicylamide at several concentrations for 3 days. As shown in Fig. 1A, salicylamide did not induce cell death. The same results were observed in time course studies (Fig. 1B). We also assessed cell proliferation following salicylamide treatment. As shown in Fig. 1C, low concentrations of salicylamide (250 μM) did not inhibit cell proliferation. However, at high concentrations, salicylamide inhibited cell proliferation (approximately 70% and 55% at 500 μM and 1,000 μM, respectively). In time course experiments, salicylamide gradually inhibited cell proliferation (Fig. 1D).

The effect of salicylamide on cell cycle progression

Next, we performed flow cytometric analysis to elucidate the effect of salicylamide on the cell cycle. As shown in Fig. 2, the percentage of cells in the G1 phase increased in the pres-
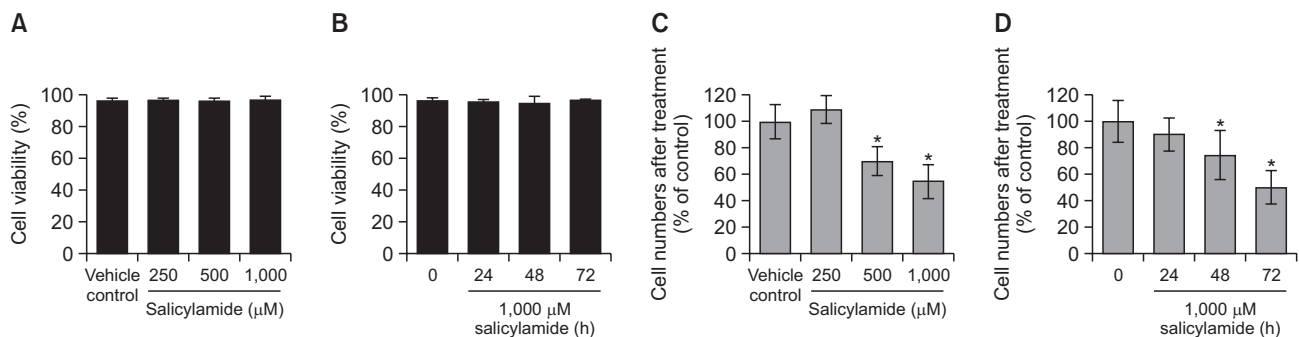

Fig. 1. Effect of salicylamide on cell viability and proliferation. B16F1 melanoma cells were treated with different doses of salicylamide for 3 days (A, C) and 1,000 μM salicylamide at the indicated time points (B, D). After treatment, cell viability and cell numbers were determined by the trypan blue exclusion test. *p<0.01 versus the control group.

ence of salicylamide. Conversely, the percentages of cells in the S and G2/M phases slightly decreased.

Salicylamide induced melanin synthesis in B16F1 melanoma cells

To elucidate the effect of salicylamide on melanin synthesis, we performed a melanin content assay. B16F1 melanoma cells were treated with or without salicylamide for 3 days and solubilized in 2 M NaOH. As shown in Fig. 3A, melanin content increased in the treated cells in a dose-dependent manner (approximately 15 pg/cell, 40 pg/cell, 70 pg/cell and 170 pg/cell for vehicle control, 250 μM, 500 μM, and 1,000 μM salicylamide, respectively). We also performed a time-course experiment at in the presence of 1,000 μM salicylamide. No pigmentation enhancement was observed after 24 h of treatment (Fig. 3B). However, melanin content increased significantly after longer treatment periods (approximately 60 pg/cell at 48 h and 160 pg/cell at 72 h of treatment). These results show that melanin formation started at least 24 h after salicylamide treatment.

The direct effect of salicylamide on mushroom and cellular tyrosinase activity

We previously reported that some NSAIDs could inhibit the monophenolase and/or diphenolase activities of mushroom tyrosinase (Sato and Toriyama, 2011). Therefore, in the cur-
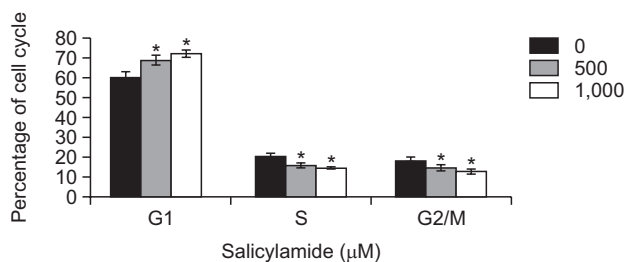
rent study, we assessed the effect of salicylamide on monophenolase and diphenolase activities. As shown in Fig. 4A, salicylamide slightly inhibited enzyme activity in a monophenolase assay (approximately 82% at 1,000 μM). However, salicylamide surprisingly enhanced diphenolase activity. As shown in Fig. 4B, diphenolase activity increased in a dose-dependent manner with up to 1,000 μM salicylamide treatment (approximately 145% at 1,000 μM). However, diphenolase activity peaked in the presence of 2,000 μM salicylamide (approximately 135% at 2,000 μM).

Next, we elucidated the direct effect of salicylamide on cellular tyrosinase. In this assay, we used the cell lysate of α-MSH-stimulated B16F1 melanoma cells. As shown in Fig. 4C, salicylamide did not affect mouse melanoma-derived tyrosinase activity.

Intracellular tyrosinase activity after salicylamide treatment (DOPA staining)

To elucidate whether salicylamide affects intracellular tyrosinase activity, we performed a DOPA staining assay. In this
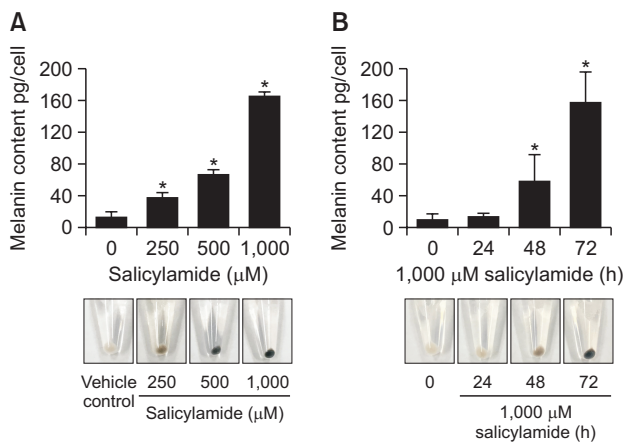

Fig. 2. Flow cytometric analysis of the cell cycle. Cells were treated with different doses of salicylamide for 3 days. After treatment, the cells were fixed, and flow cytometric analysis was performed as described in the "Materials and Methods" section. G1 phase: preparation for DNA synthesis; S phase: DNA synthesis; G2 phase; cell growth; M phase: mitosis. *p<0.01 versus the control group.

Fig. 3. Effect of salicylamide on melanin synthesis. Cells were treated with different doses of salicylamide for 3 days (A) and 1,000 μM salicylamide at the indicated time points (B). After treatment, the cell pellets were photographed, and melanin content was measured as described in the "Materials and Methods" section. *p<0.01 versus the control group.
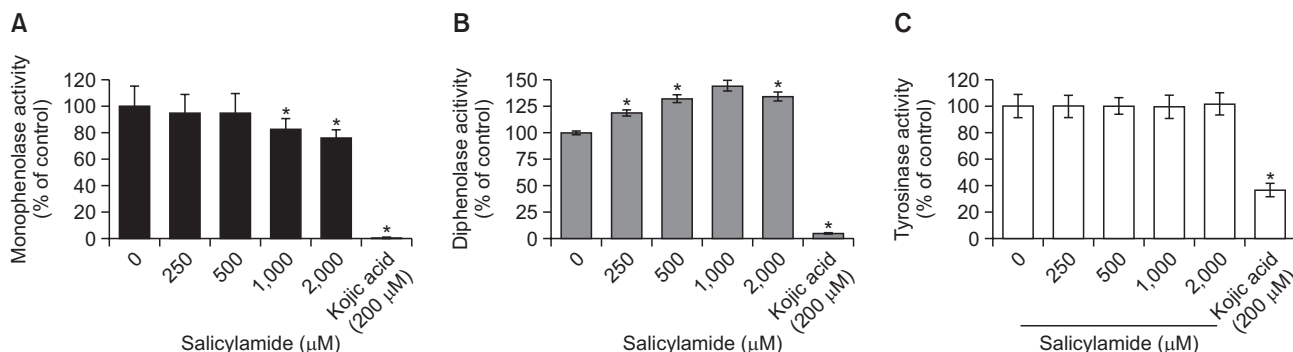

Fig. 4. Tyrosinase assays. The effect of salicylamide on monophenolase (A) and diphenolase activity (B) of mushroom tyrosinase was measured. (C) Direct effect of salicylamide on cellular tyrosinase. The results are expressed as a percentage of the control. *p<0.01 versus the control group.

assay, we treated B16F1 cells with several concentrations of salicylamide for the indicated time periods. As shown in Fig. 5, salicylamide enhanced tyrosinase activity in a time- and dose-dependent manner. Interestingly, 24 h treatment with salicylamide enhanced tyrosinase activity only at the 1,000 μM dose. These results indicate that salicylamide enhances intracellular tyrosinase activity directly and/or via transactivation of melanogenesis-related genes.

Salicylamide increased tyrosinase protein levels

We speculated that the enhancement of melanogenesis caused by salicylamide results in the activation of gene transcription and/or protein expression. Therefore, we carried out western blotting analysis. B16F1 melanoma cells were treated with or without salicylamide for 3 days. As shown in Fig. 6, salicylamide treatment significantly enhanced tyrosinase protein levels. However, the levels of other melanogenic proteins, such as TRP-1, TRP-2, and Mitf, were not changed in the presence of salicylamide.

Salicylamide enhanced tyrosinase mRNA levels

To clarify the effect of salicylamide on melanogenic gene transcription, we carried out real-time RT-PCR. As shown in Fig. 7A, tyrosinase mRNA level was dramatically enhanced after 72 h of salicylamide treatment whereas the level of other

melanogenic genes remained unchanged. We also evaluated the effect of salicylamide on *Mitf* mRNA level during shorter time periods (0.5-6 h). The results indicated that *Mitf* mRNA enhancement started at 2 h of salicylamide treatment (Fig. 7B).

DISCUSSION

We previously reported that several NSAIDs could inhibit melanin synthesis. Kumar *et al*. (2018) also investigated the effect of aspirin on melanoma metabolism and indicated that the depigmenting effect of aspirin results in PGE2 suppression. Unpredictably, we found that salicylamide activated melanin synthesis in B16 melanoma cells. Therefore, in this study, we investigated the mechanism through which salicylamide activates melanogenesis. Salicylamide did not show any cytotoxicity in B16F1 melanoma cells. However, salicylamide treatment arrested cell proliferation. To investigate the underlying mechanism, we evaluated the cell cycle of treated cells. In the presence of salicylamide, the percentage of cells in the G1 phase increased, whereas the percentages of cells in the S and G2/M phases decreased. Generally, melanogenesis inducers, such as α-MSH and IBMX, inhibit cell proliferation;
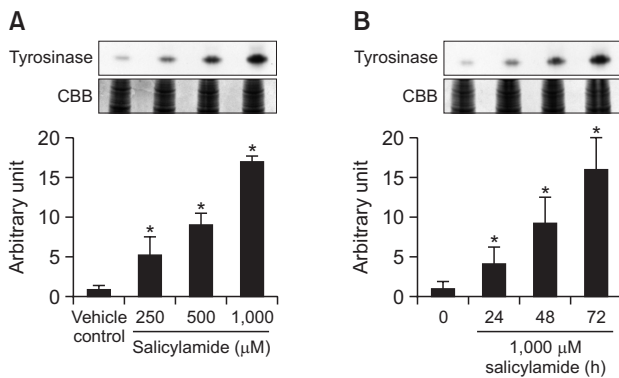
Fig. 5. DOPA staining in gels. Cells were treated with different doses of salicylamide for 3 days (A), and 1,000 μM salicylamide at the indicated time points (B). After treatment, the cell lysates were subjected to SDS-PAGE. The gels were then soaked in L-DOPA solution for analysis. *$p<0.01$ versus the control group.
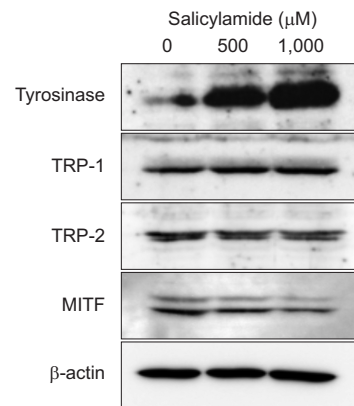
Fig. 6. Western blot analysis. Cell were treated with salicylamide for 3 days. After treatment, western blotting of tyrosinase, TRP-1, TRP-2, and Mitf was performed. The loading control was assessed using an anti-β-actin antibody.
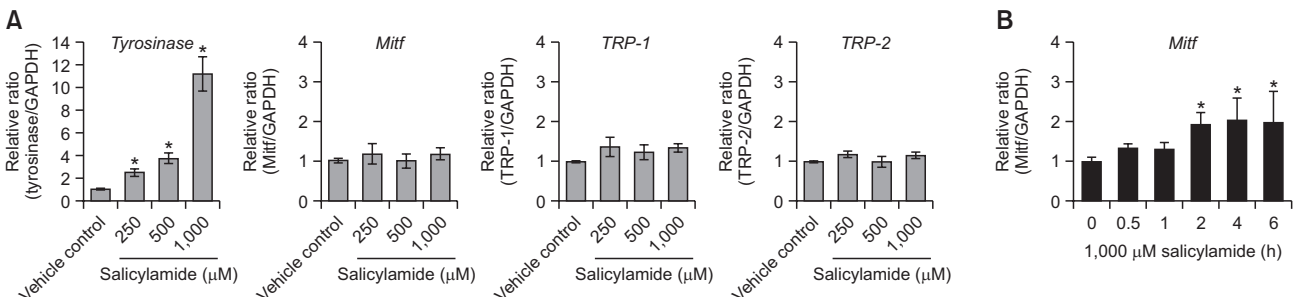
Fig. 7. Quantitative RT-PCR analysis. Cells were treated with salicylamide for 3 days (A) and 1,000 μM salicylamide at the indicated time points (B). After treatment, total RNA was extracted, and cDNA was prepared. An equivalent amount of cDNA was amplified using primers specific for tyrosinase, *TRP-1*, *TRP-2*, and *Mitf*. The results were normalized to *GAPDH* mRNA levels. *$p<0.01$ versus the control group.

thus, we speculate that salicylamide-induced cell cycle arrest results in the activation of melanogenesis. In the melanin content assay, salicylamide induced melanogenesis in a dose-dependent manner. This result indicates that salicylamide is a potential strong inducer of melanogenesis.

To investigate the melanism of salicylamide-enhanced melanin synthesis, we performed several assays. First, we assessed the effect of salicylamide on mushroom tyrosinase activity. Interestingly, salicylamide enhanced diphenolase activity directly. In contrast, monophenolase activity was slightly inhibited in the presence of salicylamide. Furthermore, we elucidated the direct effect of salicylamide on mouse tyrosinase using B16F1 melanoma cell lysate. However, salicylamide did not affect cellular tyrosinase activity. The influence of salicylamide on tyrosinase activities does not apparently contribute to the enhancement of melanin synthesis in B16F1 melanoma cells. Previously, we assessed several NSAIDs for their effects on monophenolase and diphenolase activities (Sato and Toriyama, 2011). We found that diflunisal and indomethacin inhibited tyrosinase activity. However, several NSAIDs, such as acetylsalicylic acid, ibuprofen, and diclofenac, did not show any inhibitory activity. Based on these results, we hypothesize that the effect of NSAIDs as cyclooxygenase inhibitors is not related to tyrosinase activity.

Next, we performed a DOPA staining assay in gels. In this assay, melanoma cells were treated with or without salicylamide for the indicated time periods. Thus, we can estimate the amount of active tyrosinase generated and removed by *de novo* synthesis and proteolysis, respectively. The intracellular active form of tyrosinase was enhanced in a time- and dose-dependent manner. In the melanin content assay, the cells did not show any significant enhancement 24 h after treatment. However, we confirmed that the active form of intracellular tyrosinase was up-regulated only after 24 h of salicylamide treatment. These results show that salicylamide not only increases tyrosinase enzyme activity but also induces tyrosinase expression.

To investigate the effect of salicylamide on intracellular melanogenic protein levels and gene transcription, we carried out western blotting analysis and quantitative RT-PCR, respectively. Tyrosinase is a critical enzyme that can enhance melanogenesis; therefore, this enzyme is the main target for screening of melanogenic regulatory agents. Our results indicate that salicylamide treatment increased tyrosinase mRNA and protein levels. However, other melanogenic proteins, such as Mitf, TRP-1, and TRP-2, were not affected after 72 h of salicylamide treatment. Mitf plays a pivotal role in tyrosinase gene transcription (Bertolotto *et al*., 1998; Steingrimsson *et al*., 2004). To solve this inconsistency, we assessed *Mitf* mRNA levels after salicylamide treatment for shorter periods of time. After treatment for only 2 h, *Mitf* mRNA level was increased. Therefore, salicylamide can enhance *Mitf* mRNA level and subsequent tyrosinase transcription. Salicylamide is widely used as an analgesic antipyretic drug, for which the peak blood concentration after oral co-administration with acetylsalicylic acid is 24.8 μM (Abdel-Rahman *et al*., 1991). Under our experimental conditions, the concentrations of salicylamide were much higher; however, we assume the drug would be administered topically. Moreover, the fact that salicylamide enhances melanin synthesis is an important finding with regard to drug repurposing.

Skin and hair color are directly related to beauty and at-

tractiveness. Abnormal melanin synthesis leads to several skin disorders. For example, melanin overproduction leads to melasma, freckles, and post-inflammatory hyperpigmentation. In contrast, melanin underproduction leads to vitiligo and leukoderma (Nicolaidou and Katsambas, 2014). Vitiligo is one of the most common depigmentation disorders that affects an estimated 0.5%-1% of the world population (Ezzedine *et al*., 2012; Niu and Aisa, 2017). Several hypotheses have been proposed to explain the causes of this disorder, the most prominent being accumulation of oxidative stress, an autoimmune disorder, and genetic aspects (Alikhan *et al*., 2011). To date, several compounds and plant extracts have been reported to stimulate melanin synthesis. For example, Kim *et al*. (2020) reported that an ethanolic extract of the root of *Polygonum multiflorum* Thunb. stimulated melanin synthesis by inducing Mitf expression in B16F10 melanoma cells. Ullah *et al*. (2020) demonstrated that the antibiotic fosfomycin enhanced melanogenesis through the upregulation of c-Jun N-terminal kinases and p38 pathways, which are involved in the regulation of Mitf. Currently, the common treatment for vitiligo is skin exposure to UVB (Scherschun *et al*., 2001). However, UV is harmful to the skin. Thus, screening for new compounds with an anti-whitening effect is very important.

In conclusion, our results indicate that salicylamide can induce melanin synthesis rapidly, mainly by upregulating tyrosinase expression via *Mitf* transcription. Therefore, this study demonstrates that salicylamide may be a useful inducer of melanogenesis in cosmetics (including for hair graying) or for the medical treatment of hypopigmentation disorders.

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