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Nodakenin inhibits melanogenesis via the ERK/MSK1 signaling pathway

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The aim of the present study was to investigate the potential inhibitory effects of nodakenin, a coumarin glucoside derivative from the root extract of *Angelica gigas* Nakai (AGN), on melanogenesis and its underlying mechanisms in B16F10 melanoma cells. The inhibitory effects of nodakenin on melanogenesis were evaluated by determining melanin contents and tyrosinase activity in α-melanocyte stimulating hormone (α-MSH)-treated B16F10 melanoma cells. The mechanisms associated with the anti-pigmentation effect of nodakenin were investigated by quantitative real-time PCR and immunoblotting analysis. Using the UVB-irradiated conditioned media culture system and UVB-irradiated co-cultivation system of HaCaT keratinocytes and B16F10 melanoma cells mimicking *in vivo* melanin biosynthesis, the effect of nodakenin on melanin production was evaluated. Melanin content analysis showed that nodakenin decreased cellular melanin biosynthesis in α-MSH-treated B16F10 cells. Immunoblotting revealed that CREB phosphorylation, MITF, a mastering transcription factor of melanogenesis and its downstream genes tyrosinase, tyrosinase-related protein 1, and tyrosinase-related protein 2 were downregulated by nodakenin in a dose-dependent manner. Interestingly, nodakenin did not affect the phosphorylation of PKA and p38 MAPK but the phosphorylation of ERK1/2 and MSK1. In addition, the inhibition of melanin accumulation by nodakenin in the UVB-irradiated conditioned media culture system and UVB-irradiated co-cultivation system of HaCaT and B16F10 cells suggests that nodakenin has potential as an anti-pigmentation activity. These data suggest that nodakenin inhibits the melanogenesis in B16F10 cells by interfering the ERK/MSK1/CREB axis and thus preventing MITF expression.

1. Introduction

Melanin, a pigment which determines the color of individual skin and hair, is synthesized by melanocytes and transferred to keratinocytes as the form of melanosomes and has a role of protecting the tissues from the ultraviolet rays (UV)-induced cell damage by removing reactive oxygen species (ROS) (D'alba and Shawkey 2019; Marks and Seabra 2001; Slominski et al. 2004). This process is associated with catalytic mechanisms of melanogenic enzymes such as tyrosinase (Tyr), tyrosinase-related protein-1 (Tyrp1), and tyrosinase-related protein-2 (Tyrp-2) (Kadekaro et al. 2012; Schallreuter et al. 2008). In particular, tyrosinase is one of the key enzymes in melanin biosynthesis (Cooksey et al. 1998). Tyrosinase enzymatically converts L-tyrosine into L-dihydroxyphenylalanine (L-DOPA), which is transformed to L-dopaquinone, and these compounds act as the precursor of melanin (Cooksey et al. 1998; Slominski et al. 2004). Additionally, catalytic actions of Tyrp-1 and Tyrp-2 are essential for synthesizing eumelanin, a type of melanin that represents black and deep-brown colors in tissues (Videira et al. 2013).

Melanogenesis comprises a series of processes for synthesis and delivery of melanin (Bonaventure et al. 2013). UV radiation is the main physiological stimulus in human melanogenesis (Passeron et al. 2005). UV exposure to keratinocyte induces several cellular changes including activation of p53 (Cui et al. 2007; Hyter et al. 2013). Activated p53 stimulates the promoter of pro-opiomelanocortin (POMC), the precursor for melanotropic, corticotropic, and opioid peptides such as α-melanocyte-stimulating hormone (α-MSH), ACTH, and other released peptides (Cui et al. 2007; Lim et al. 2016; Slominski et al. 2000, 2004). The POMC-derived peptide then binds to melanocortin 1 receptor (MC1R) on the surface of melanocyte, one of the G protein-coupled receptors (GPCR) (Slominski et al. 2004; Tsatmali et al. 2002). Activated MC1R leads to the accumulation of cyclic AMP and phosphorylation of protein kinase A (PKA) followed by the activation of CREB (cAMP response element-binding protein)/ Microphthalmia-associated transcription factor (MITF) signaling pathway in melanocyte (Edelman et al. 1987; Shibahara et al. 2000). MITF acts as the major transcription factor of tyrosinase, tyrosinase-related proteins, and itself (Levy et al. 2006; Yasumoto et al. 1994). As another signaling mechanism, it has been reported that the accumulation of cyclic adenosine monophosphate (cAMP) also triggers melanogenesis by activating extracellular signal-regulated protein kinase (ERK) through Ras-Raf-MEK signaling pathway in melanocytes (Busca et al. 2000). Furthermore, previous studies have been demonstrated that the activated ERK promotes the phosphorylation of stress-activated kinase 1 (MSK1), and the phosphorylated MSK1 directly activates CREB for regulating the melanogenesis (Busca et al. 2000; Roux and Blenis 2004; Tagashira et al. 2015).

A substance derived from medicinal plants is referred to as phytochemical and the substances are important in antibacterial, vascular formation, inflammatory reactions, cell proliferation, vascular production promotion and tissue regeneration (Pham et al. 2020). These have been used for food or medicinal purposes for a long time, and their safety and medicinal effectiveness have been proven and reported (Martel et al. 2020; Mishra and Tiwari 2011). Nodakenin is a coumarin component that initially derived from the root extract of *Angelica gigas* Nakai (AGN) (Jeong et al. 2015). Nodakenin has been well known for its anti-inflammatory (Lee et al. 2017; Liao et al. 2021; Lim et al. 2021), neurogenic (Gao et al. 2015), and neuroprotective (Li et al. 2020) effects. It has been also

reported that nodakenin alleviates the inflammation on allergic asthma (Xiong et al. 2014) and atopic dermatitis-like skin lesions in mice model (Park et al. 2014). *Angelica gigas* Nakai (AGN) is a medicinal herb known as 'Korean danggui' and its root extracts have been reported to contain various coumarin compounds including nodakenin, decursin, decursinol, and decursinol angelate (Jeong et al. 2015). Most of these coumarin compounds have been reported to have anti-cancer, antioxidant and antibacterial effects (Ahn et al. 2019). Also, previous studies have demonstrated the anti-pigmentation effects of decursin, ferulic acid, and chlorogenic acid in B16 melanoma cells (Li et al. 2014; Maruyama et al. 2018; Park et al. 2018). However, the effect of nodakenin on melanogenesis has not ben studied. Therefore, the present study aimed to investigate this and to elucidate its cellular signaling mechanism.

2. Investigations and results

2.1. Nodakenin inhibits the melanin production in α-MSH-induced B16F10 melanoma cells

Nodakenin is a glucoside that has a coumarin chemical structure (Fig. 1A). Prior to examine whether nodakenin affects melanogenesis, we first determined the dose range of nodakenin that causes cytotoxicity in B16F10 melanoma cells by WST-1 assay. B16F10 cells were treated with nodakenin at concentrations of 1, 5, 10, 25, 50, 75 and 100 μM for 24 h. As shown Fig. 1B, cell viability was not significantly affected in nodakenin-treated B16F10 cells at concentrations below 25 μM ($p < 0.05$). Cell cytotoxicity was observed at a concentration of above 50 μM nodakenin compared to the control (Fig. 1B). Subsequently, to test whether nodakenin affects melanin synthesis, we investigated the intracellular melanin contents in nodakenin-treated B16F10 cells. Cells were co-treated with nodakenin (10 and 25 μM) and α-MSH for induction of melanogenesis for 48 h and then cellular melanin content assay was performed. Arbutin was used as positive control for this assay. As shown in Fig. 1C, nodakenin significantly reduced α-MSH-induced melanin contents in a dose-dependent manner. The melanin contents treated with 25 μM of nodakenin was lower than those treated with arbutin, which was used as the positive control (Fig. 1C). Since it has been known that cellular tyrosinase activity, which converts L-DOPA to L-dopachrome, is a key enzymatic process for melanogenesis (Cooksey et al. 1998), we analyzed the cellular tyrosinase activity in nodakenin-treated B16F10 cells by measuring the absorbance of 450 nm that could detect the amount of L-dopachrome. Our data showed that nodakenin inhibited cellular tyrosinase activity in a dose-dependent manner (Fig. 1D). To determine whether nodakenin affects the tyrosinase enzymatic activity directly, *in vitro* mushroom tyrosinase activity assay was also performed. Figure 1E showed that nodakenin had little or no inhibitory effect on mushroom tyrosinase activity. These results collectively indicated that the decrease of the melanin production in nodakenin-treated B16F10 cells was associated with the reduced expression of intracellular tyrosinase, but not affected by tyrosinase own activity.

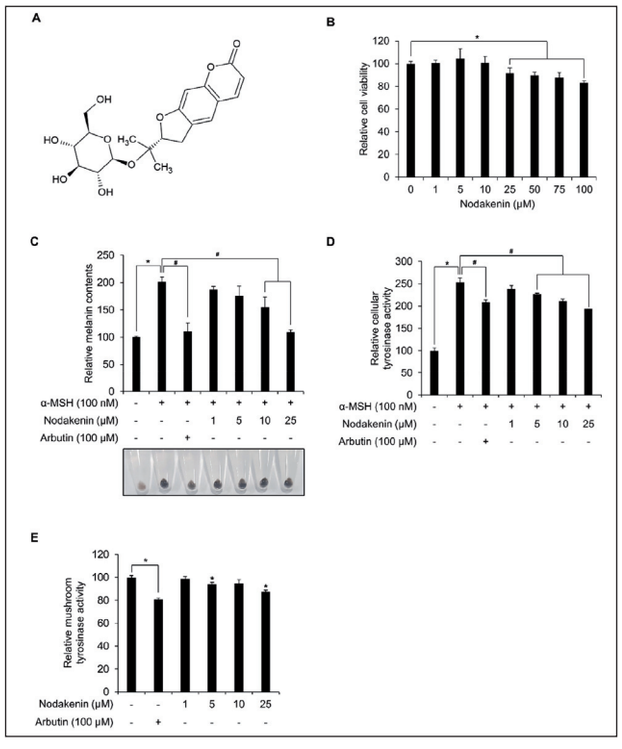

Fig. 1: Effect of nodakenin on melanogenesis in B16F10 melanoma cells. (A) Chemical structure of nodakenin. (B) B16F10 cells were treated with the indicated concentrations of nodakenin (1–100 μM) for 24h. Cell viability was measured using WST-1 assay. (C) The melanin contents of B16F10 cells treated with α-MSH (100 nM) and nodakenin (1–25 μM) for 48 h was observed via the optical density (450 nm). Relative melanin contents were determined by assessing the amount of dopachrome formed. (D) The cellular tyrosinase activity of B16F10 cells treated with α-MSH (100 nM) and nodakenin (1–25 μM) for 24 h was observed via the optical density (450 nm). Relative cellular tyrosinase activities were determined by measuring dopachrome formation from the L-DOPA substrate. (E) The direct effect of treatment of nodakenin (1–25 μM) on tyrosinase activity was assessed using mushroom tyrosinase in a cell-free system. Relative mushroom tyrosinase activities were determined by measuring the dopachrome formation from the L-DOPA substrate. Arbutin was applied as a positive control. The results are expressed as percent relative to the control. The data are presented as the mean ± SD of three independent experiments. Significance was determined using a one-way ANOVA followed by Tukey's test. *$p < 0.05$ compared with the vehicle treated group. #$p < 0.05$ compared with α-MSH treated group. Values of $p < 0.05$ were considered to be statistically significant.

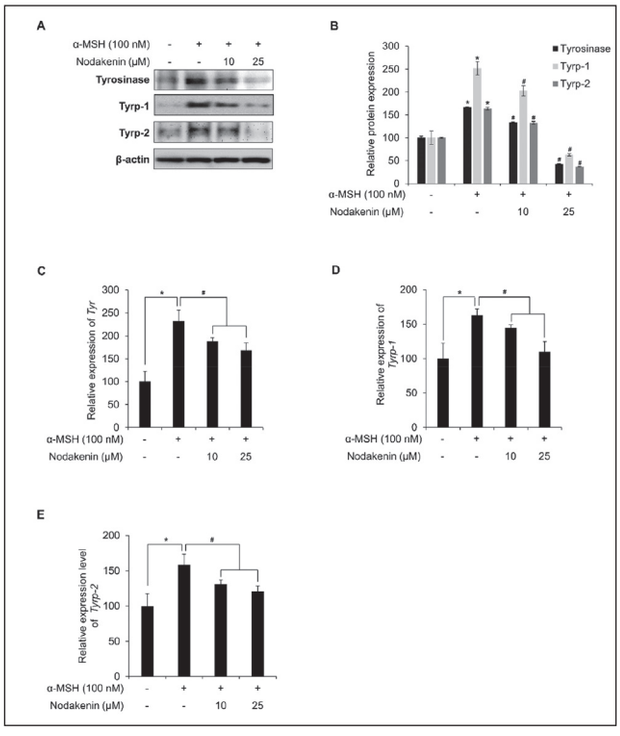

Fig. 2: Effect of nodakenin on expressions of tyrosinase and its related proteins in B16F10 melanoma cells. (A) B16F10 cells were co-cultured with α-MSH (100 nM) and nodakenin (10 and 25 μM) for 24 h. The expressions of tyrosinase, Tyrp-1, and Tyrp-2 were analyzed using immunoblotting with the specific antibodies. The Image-J program was used to quantify the tyrosinase, Tyrp-1, and Tyrp-2 blots and equal protein loading was confirmed by normalizing with β-actin antibodies. (B) Quantification of protein expression by scanning densitometry in (A). B16F10 cells were co-cultured with α-MSH (100 nM) and nodakenin (10 and 25 μM) for 9 h. The mRNA expressions of (C) *Tyrosinase* (*Tyr*), (D) *Tyrp-1*, and (E) *Tyrp-2* were analyzed using qRT-PCR with the specific primers. Each result was normalized by *Gapdh* for qRT-PCR assay. The data are presented as the mean ± SD of three independent experiments. Significance was determined using a one-way ANOVA followed by Tukey's test. *$p < 0.05$ compared with the vehicle treated group. #$p < 0.05$ compared with α- MSH treated group. Values of $p < 0.05$ were considered to be statistically significant.

2.2. Nodakenin inhibits the cellular expression of tyrosinase and its related proteins via down-regulation of MITF

Since the melanin production was significantly suppressed by nodakenin, we next examined whether nodakenin alleviated the expressions of melanogenic enzymes including tyrosinase, tyrosinase-related protein 1 (Tyrp-1) and tyrosinase-related protein 2 (Tyrp-2) by immunoblotting. Following treatment with nodakenin in B16F10 cells for 24 h, the expression levels of tyrosinase, Tyrp-1 and Tyrp-2 were decreased compared to the control (Fig. 2A-B). Next, quantitative RT-PCR analysis revealed that nodakenin downregulated the transcriptional expressions of tyrosinase, Tyrp-1, and Tyrp-2 genes (Fig. 2C-E). Since Mitf has been reported to act as a master transcription factor for expression of Tyr, Tyrp-1, and Tyrp-2 on melanin biosynthesis process (Yasumoto et al. 1994), we further examined whether nodakenin affects the expression of Mitf. Immunoblotting indicated that the expression of Mitf was significantly decreased in nodakenin-treated B16F10 cells (Fig. 3A-C).

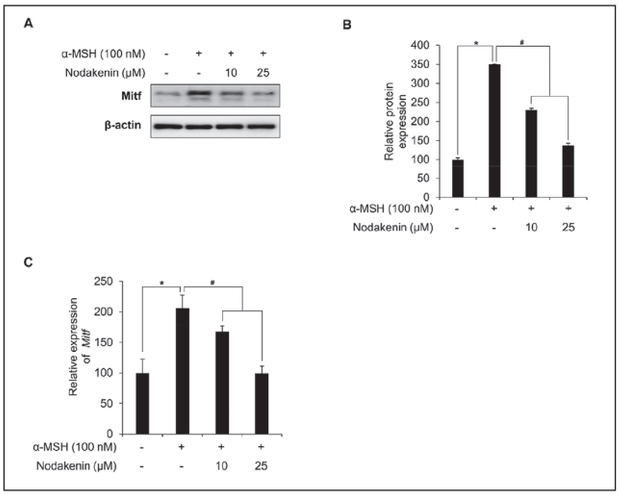

Fig. 3: Effect of nodakenin on expression of MITF in B16F10 melanoma cells. (A) B16F10 cells were co-cultured with α-MSH (100 nM) and nodakenin (10 and 25 μM) for 24 h. The expression level of Mitf protein were analyzed using immunoblotting with the specific antibodies. (B) Quantification of protein expression by scanning densitometry in (A). The Image-J program was used to quantify the Mitf blot and equal protein loading was confirmed by normalizing with β-actin antibodies. (C) B16F10 cells were co-cultured with α-MSH (100 nM) and nodakenin (10 and 25 μM) for 9 h. The mRNA expression of *Mitf* was analyzed using qRT-PCR with the specific primers. The result was normalized by *Gapdh* for qRT-PCR assay. The data are presented as the mean ± SD of three independent experiments. Significance was determined using a one-way ANOVA followed by Tukey's test. *$p < 0.05$ compared with the vehicle treated group. #$p < 0.05$ compared with α- MSH treated group. Values of $p < 0.05$ were considered to be statistically significant.

2.3. Nodakenin downregulates the phosphorylation of CREB by regulating ERK/MSK1 signaling pathway

It has been reported that α-MSH can induce the expression of Mitf through PKA/CREB signaling pathway in melanocyte (Shibahara et al. 2000), and we subsequently investigated whether nodakenin affects the phosphorylation of CREB and PKA proteins in α-MSH treated-B16F10 cells by immunoblotting. Figures 4A and 4B show the phosphorylated form of CREB at serine 133 residue and PKA at serine 197 residue were markedly elevated in α-MSH treated-B16F10 cells. The phosphorylation level of CREB was restrained by nodakenin treatment in α-MSH treated-B16F10 cells (Fig. 4A-B). By contrast, the phosphorylation level of PKA was not affected by nodakenin treatment (Fig. 4A-B). We further tested whether the activities of ERK and p38, which are known to induce the activation of CREB through the regulation of MSK1, are affected by nodakenin in α-MSH treated-B16F10 cells. Figures 5A and 5B showed that the phosphorylation of ERK was significantly decreased by nodakenin in a dose-dependent manner; however,

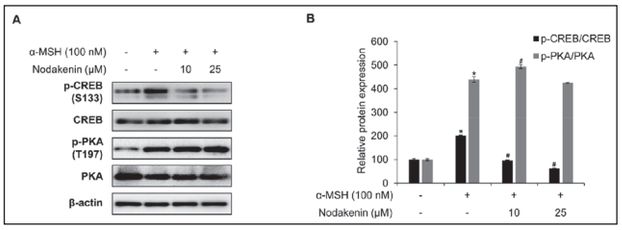

Fig. 4: Effect of nodakenin on PKA/CREB pathway in B16F10 melanoma cells. B16F10 cells were co-cultured with α-MSH (100 nM) and nodakenin (10, 25 μM) for 12 h. (A) Western blotting was conducted to investigate the changes in the phosphorylation of CREB and PKA proteins. Each result was normalized by β-actin for immunoblotting. (B) Quantification of protein expression by scanning densitometry in (A). The Image-J program was used to quantify the p-CREB and p-PKA blots and equal protein loading was confirmed by normalizing with the total protein CREB and PKA blots, respectively. The data are presented as the mean ± SD of three independent experiments. Significance was determined using a one-way ANOVA followed by Tukey's test. *$p < 0.05$ compared with the vehicle treated group. #$p < 0.05$ compared with α- MSH treated group. Values of $p < 0.05$ were considered to be statistically significant.

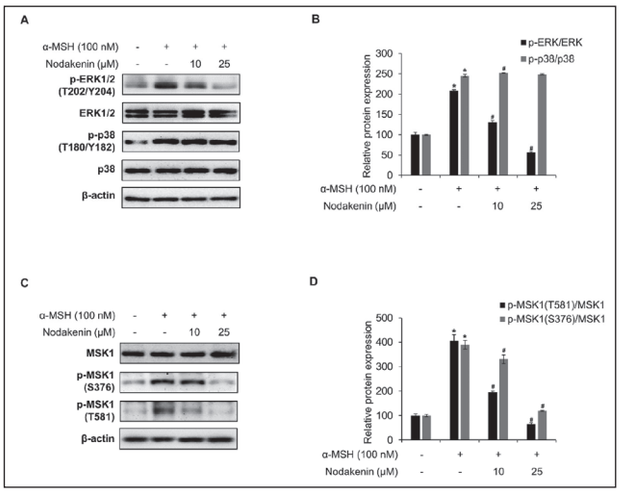

Fig. 5: Effect of nodakenin on ERK/MSK1 pathway in B16F10 melanoma cells. B16F10 cells were co-cultured with α-MSH (100 nM) and nodakenin (10, 25 μM) for 6 h. Western blotting was conducted to investigate the changes in the phosphorylation of (A) ERK1/2, p38 and (C) MSK1 proteins. Each result was normalized by β-actin for immunoblotting. (B) Quantification of protein expression by scanning densitometry in (A). The Image-J program was used to quantify the p-ERK1/2, p-p38, p-MSK1(Thr581) and p-MSK1(Ser376) blots and equal protein loading was confirmed by normalizing with the total protein ERK1/2, p38, and MSK1 blots, respectively. The data are presented as the mean ± SD of three independent experiments. Significance was determined using a one-way ANOVA followed by Tukey's test. *$p < 0.05$ compared with the vehicle treated group. #$p < 0.05$ compared with α- MSH treated group. Values of $p < 0.05$ were considered to be statistically significant.

there was no significant decrease on phosphorylation of p38 in nodakenin-treated B16F10 cells. Therefore, we further investigated whether nodakenin affects MSK1/CREB signaling pathway, an alternate melanogenesis signaling mechanism. Immunoblotting demonstrated that nodakenin effectively downregulated the phosphorylation of MSK1 at threonine 581 and serine 376 residues in α-MSH treated-B16F10 cells (Fig. 5C-D). Our data indicated that nodakenin inhibits the CREB phosphorylation via MSK1 signaling pathway known for an alternate activator of CREB proteins. These results suggested that nodakenin has an inhibitory function on the melanin biosynthesis process via ERK/MSK1/CREB axis.

2.4. Nodakenin suppresses the melanin production in UVB-irradiated conditioned media culture system and keratinocyte and melanocyte co-culture system

To further evaluate the effect of nodakenin under physiological conditions mimicking the process of melanin biosynthesis in the skin, we analyzed the melanin production in UVB-irradiated

conditioned media culture system and keratinocyte and melanocyte co-culture system. To perform a UVB-irradiated conditioned media culture system, HaCaT cells were seeded for 24 h and irradiated with UVB (10 mJ/cm^2). After 24 h of incubation, the conditioned media was added to B16F10 cells with nodakenin (10 and 25 μM) or arbutin for 48 h. As shown in Fig. 6A, nodakenin decreased the melanin contents in B16F10 cells induced melanogenesis by UVB-irradiated conditioned media. Additionally, HaCaT and B16F10 cells co-culture system was performed to investigate the effect of nodakenin. HaCaT and B16F10 cells were co-cultured for 24 h and irradiated to 10 mJ/cm^2 of UVB. After incubation for 24 h, the cells were treated with nodakenin or arbutin for 48 h. As a result, nodakenin suppressed the melanin production boosted by UVB irradiation. The inhibitory effect at a concentration of 25 μM nodakenin was higher than that of arbutin treatment (100 μM) used as a positive control (Fig. 6B). These results showed the potential of nodakenin to inhibit melanin biosynthesis in a physiological environment.

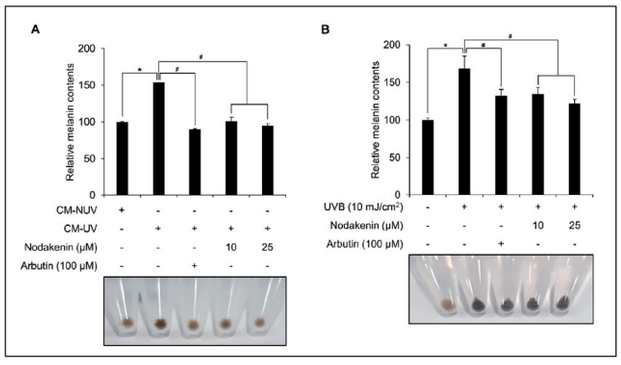

Fig. 6: Effect of Nodakenin on melanogenesis in HaCaT-B16F10 cells co-culture system. (A) HaCaT keratinocytes were seeded and irradiated with UVB (10 mJ/cm^2). B16F10 cells were co-cultured with nodakenin (10 and 25 μM) and the media obtained from HaCaT keratinocytes, which referred to as conditioned media. (B) HaCaT and B16F10 cells were co-cultured for 24 h and irradiated to 10 mJ/cm^2 of UVB. The cells were treated with nodakenin (10 and 25 μM) following incubation for 24 h. The results of both (A) and (B) melanin contents were observed via the optical density (450 nm). Arbutin was applied as a positive control. The results are expressed as percent relative to the control. The data are presented as the mean ± SD of three independent experiments. Significance was determined using a one-way ANOVA followed by Tukey's test. *$p < 0.05$ compared with the vehicle treated group. #$p < 0.05$ compared with UVB-irradiated group. Values of $p < 0.05$ were considered to be statistically significant. CM-UV: UV-irradiated conditioned media; CM-NUV: UV-nonirradiated conditioned media.

3. Discussion

Nodakenin is one of the major coumarin glucoside derivatives isolated from *Angelica gigas* Nakai (AGN). AGN, referred to 'Korean danggui', is commonly used as herbal medicine throughout the regions of Korea (Ahn et al. 2019). Notably, the root extract of AGN has been reported to possess neuroprotective activities by alleviating the ischemic injury in the transient middle cerebral artery occlusion (tMCAO)-induced mouse model and on transient focal cerebral ischemia in rats (Oh et al. 2015). Also, it has been reported that AGN root extract induces hair growth by controlling inflammatory cytokines in mouse dorsal skin model (Lee et al. 2020). Coumarin is a type of phytoalexin commonly known to act as an antimicrobial and antioxidant for plant defense reactions (De Souza et al. 2005; Kostova et al. 2011). Similarly, the antibacterial and anticancer activities of coumarin derivatives have been identified previously (De Souza et al. 2005; Lacy and O'kennedy 2004; Venkata Sairam et al. 2016). Nodakenin also has been found to remarkably exert anti-inflammatory effect in LPS-induced inflammatory macrophage cells (Rim et al. 2012), liver injury mice model (Lim et al. 2021), and allergic inflammatory mast cells (Lee et al. 2017). In addition, it has been also demonstrated that the topical application of nodakenin inhibits DNCB-induced atopic dermatitis-like symptoms in ICR mice model (Park et al. 2014). Recently, several substances such as decursin (Park et al. 2018), ferulic acid (Maruyama et al. 2018), chlorogenic acid (Li et al. 2014), and demethylsuberosin (Kim et al. 2014) contained in the AGN root extract (Jeong et al. 2015) have been reported to regulate melanogenic response as well as the extract (Lv et al. 2007). However, it has not been studied whether nodakenin is involved in melanogenesis.

In this study, we demonstrated the anti-melanogenic effect of nodakenin in B16F10 murine melanoma cells. By measuring the melanin contents in α-MSH induced B16F10 cells, we determined that nodakenin inhibited the synthesis of melanin pigments in a dose-dependent manner (Fig. 1C). Tyrosinase is an essential enzyme to catalyze the synthesis of melanin, and its cellular activity and amounts are closely involved in melanogenesis (Bentley et al. 1994; Chan et al. 2014). We identified the inhibition on cellular tyrosinase activity following nodakenin treatment (Fig. 1D). However, mushroom tyrosinase activity in a cell-free system was not significantly affected by nodakenin treatment (Fig. 1E). These results indicated that the decrease in the melanin synthesis was due to the regulation of cellular tyrosinase by nodakenin, not directly to the inhibition of tyrosinase's own activity. It has been known that tyrosinase and its related proteins are essential enzymes for melanin synthesis (Schallreuter et al. 2008). Our data show that nodakenin decreased the expressions of tyrosinase, Tyrp-1, and Tyrp-2 proteins (Fig. 2A-B). In addition, we examined the inhibition of the transcriptional expression of tyrosinase, Tyrp-1, and Tyrp-2 following the treatment with nodakenin by qRT-PCR assay (Fig. 2C-E). Mitf acts as the transcription factor on multiple cellular processes including melanogenesis, differentiation, proliferation, and cell survival. In melanogenesis, previous studies have demonstrated that the expressions of tyrosinase and tyrosinase-related protein-1 (Tyrp-1), and 2 (Tyrp-2), which are the major melanogenic enzymes, are importantly determined by the expression and activity of Mitf in melanocyte (Garcia-Borron et al. 2014). We further analyzed the effect of nodakenin on expression of Mitf and it was downregulated by nodakenin on both transcription and translation levels (Fig. 3). The results indicated that tyrosinase and its related gene expressions might be regulated by a Mitf-dependent pathway. It has been studied that the recruitment of the CREB at multiple binding sites of Mitf promoter regions triggers Mitf transcriptional activity (Shibahara et al. 2000). When keratinocytes are exposed to UVB in epidermis, α-MSH is secreted from keratinocytes through a series of processes and can stimulate melanogenesis by binding to MC1R as a ligand (Slominski et al. 2004). Then, α-MSH-stimulated MC1R activates adenylate cyclase, upregulating the cAMP levels and, in turn, induces the activation/phosphorylation of PKA and CREB proteins (Edelman et al. 1987; Shibahara et al. 2000). Our data show that the treatment of nodakenin significantly inhibits the phosphorylation of CREB and further leads to the downregulation of the Mitf transcription (Fig 4A-B). Contrary to our expectation, the phosphorylation level of PKA was not affected by the nodakenin treatment in α-MSH-induced melanoma cells indicating that another regulatory pathway was involved in the phosphorylation and activation of CREB protein, but not PKA (Fig. 4A-B).

Previous studies have revealed that CREB phosphorylation can be also mediated by mitogen-activated protein kinases (MAPKs), which include ERK and p38 (Gu et al. 2014; Yamaguchi et al. 2007). MC1R, a receptor binds with α-MSH, can induce the cAMP-stimulated activation of ERK via the regulation of Ras/Raf/MEK signaling pathway. This process was generated from the PKA-independent/MAPK signaling pathway in melanocyte (Englaro et al. 1995; Goldsmith and Dhanasekaran 2007). Notably, it has revealed that ERK and p38 regulate the phosphorylation of CREB and then Mitf expression via MSK1 activation (Busca et al. 2000). Conversely, other report has shown that activated ERK directly stimulates the degradation of Mitf proteins. However, this study was carried out in spingosin-1 phosphate (S1P)-induced melanocytes (Kim et al. 2003). Even though it may seem to need the additional in-depth discoveries for ERK signaling pathway on melanogenesis, our data showed that the

phosphorylation of ERK was remarkably decreased by nodakenin, whereas that of p38 was not affected (Fig. 5A-B). Also, there is evidence that nodakenin suppresses the phosphorylation of ERK in the liver tissue of LPS-induced liver injury mice (Lim et al. 2021). Therefore, Figure 5A and 5B show that the anti-pigmentation effect following nodakenin treatment in B16F10 cells was involved in the suppression of ERK activation. Previous studies have shown that ERK induces the phosphorylation on Thr581 or Ser360 residue of MSK1 and this process leads to the autophosphorylation on Ser 367, which is required for MSK1 activation (Mccoy et al. 2005, 2007 2007). Recently, it has been reported that the phosphorylation of CREB was decreased as the results of the inhibition of MSK1 phosphorylation on the sites of Thr581 and Ser376 residues in UV-induced normal human melanocytes (Tagashira et al. 2015). We further investigated that nodakenin suppressed the phosphorylation of MSK1 on both Thr581 and Ser376 phospho-sites, indicating that the inhibitory effect on CREB activation by nodakenin was directly associated with ERK/MSK1 pathway (Fig. 5C-D).

Under physiological conditions, melanogenesis is mainly triggered by the irradiation of UV in the epidermis for the purpose of protecting the tissues from cell stress (Ichihashi et al. 2003; Passeron et al. 2005). Keratinocytes surround each melanocyte in mammalian skin, and influence melanocyte proliferation and melanin production (Lin and Fisher 2007; Seiberg 2001). Melanin pigments produced by melanocytes are transferred to neighboring keratinocytes as the form of melanosome and accumulated on the upper surface of nuclear membrane of keratinocytes (Delevoye 2014; Kamada et al. 2004). When UV irradiates to the epidermis, keratinocytes directly stimulate melanogenesis to melanocytes by secretion of the intracellular molecules including α-MSH (Tadokoro et al. 2005; Videira et al. 2013). As a major stimulator of melanocytes, α-MSH binds to MC1R in a dominant manner over other agonists (Luger et al. 1997). To confirm whether nodakenin has the inhibitory effect in physiological condition mimicking UV-irradiated keratinocyte-mediated melanogenesis, we performed the conditioned media system obtained from UVB-irradiated HaCaT keratinocytes (Fig. 6A) and co-culture system of UVB-irradiated HaCaT keratinocytes and B16F10 melanoma cells (Fig. 6B). Our data showed that nodakenin significantly inhibits the melanogenesis in both physiological condition-mimicking systems (Fig. 6A-B). It seems that additional *in vivo* studies such as those on the mouse model or 3D-reconstucted human pigmented epidermis (3D-RHPE) model or on the mouse skin model are needed to validate the anti-pigmentation effect of nodakenin in human skin and those studies are currently underway.

In conclusion, the above results suggested that nodakenin can significantly suppress melanogenesis in α-MSH-induced melanoma cells and physiological condition-mimicking systems. Moreover, we showed that the inhibitory activity of nodakenin was associated with the regulation of the ERK/MSK1 signaling pathway (Fig. 7). Based on these results, nodakenin could be a potential agent for the treatment of hyper-pigmentation, protecting the skin tissue from UV irradiation.

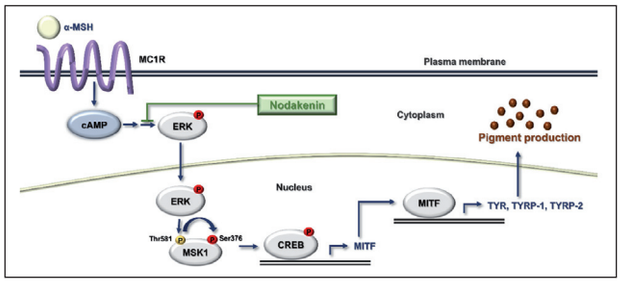

Fig. 7: Schematic model depicting the modulation of melanogenesis through the ERK/MSK1 pathway regulated by Nodakenin. Nodakenin inhibits melanogenesis by regulating the activation of CREB via the ERK/MSK1 signaling pathway

4. Experimental

4.1. Reagents and antibodies

Nodakenin, L-DOPA and mushroom tyrosinase were purchased from Sigma Aldrich (St. Louis, Missouri, USA). The primary antibodies for β-actin, Mitf, tyrosinase, Tyrp-1, and Tyrp-2 were purchased from Santa Cruz Biotechnology (Dallas, Texas, USA) and primary antibodies for PKA, p-PKA (Thr197), CREB, p-CREB (Ser133), ERK1/2, p-ERK1/2 (Thr202/ Tyr204), p38, p-p38 (Thr180/Tyr182), MSK1, p-MSK1 (Ser376), and p-MSK1 (Thr581) were purchased from Cell signaling Technology (Danvers, USA). Horseradish peroxidase (HRP)-conjugated secondary anti-rabbit and anti-mouse antibodies were obtained from Cell signaling Technology (Danvers, USA).

4.2. Cell culture

B16F10 mouse melanoma cells and HaCaT keratinocytes were cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, USA) supplemented with 10% fetal bovine serum (Biowest, Nuaillé, France) and 1% penicillin-streptomycin (Gibco, Grand Island, USA) in a 37 °C incubator under the humidified atmosphere with 5% CO_2.

4.3. Cell viability assay

Cell viability was assessed by the water-soluble tetrazolium salt (WST-1) assay using Ez-Cytox (Dogen, Seoul, Korea) reagent as manuscript instructions. Briefly, the B16F10 cells (3.5×10^3 cells/well) were seeded and further cultured for 24 h. Then, cells were treated with various concentrations of nodakenin ranging from 1 to 100 μM. Following 24 h incubation, 10 μl of the water-soluble tetrazolium salt (WST-1) solution was added to each well and incubated for 30 min at 37 °C. The absorbance was analyzed at 450 nm by a Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Winooski, USA).

4.4. Assessment of melanin contents

For assessing melanin contents, the B16F10 cells in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin were seeded at a density of 2.5×10^5 cells per wells and treated with α-MSH, and nodakenin (1-25 μM) or arbutin (100 μM) as indicated in the figures. Cell pellets were completely solubilized with 1 N NaOH lysis buffer and boiled at 95 °C for 30 min. The mean absorbance at 450 nm was measured using a Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Winooski, USA).

4.5. Tyrosinase activity assay

To measure the cellular tyrosinase activity in B16F10 melanoma cells, the cells were seeded (2.5×10^5 cells/well) and treated as indicated in the figures. After incubation for 24 h, the cells were lysed with radioimmunoprecipitation assay (RIPA) buffer and centrifuged at 15,520 × g for 30 min at 4 °C. The supernatant was reacted with 10 mM L-DOPA and incubated at 37 °C for 1 h. *In vitro* mushroom tyrosinase activity was examined as previous study (Lee et al. 2018). Briefly, each sample with different concentrations of nodakenin was added to 10 mM L-DOPA and incubated for 30 min at 100 °C. The relative tyrosinase activity was assessed spectrophotometrically at 450 nm using Synergy™ HTX Multi-Mode Microplate Reader (BioTek, Winooski, USA) and corrected from the standard curve obtained by BCA assay.

4.6. Quantitative polymerase chain reaction (qPCR)

Total RNAs were extracted from cells using Ribo-EX (Geneall, Seoul, Korea). One microgram of total RNAs were reverse-transcribed into cDNA with the Molony Murine Leukemia Virus reverse transcriptase according to the manufacturer's protocol (Invitrogen, Waltham, USA). The expressions of Gapdh, Mitf, tyrosinase, Tyrp-1, and Tyrp-2 genes were quantitatively evaluated with SYBR™ green PCR master mix (Thermo Fisher Scientific, Waltham, USA) using a Step OnePlus Real-Time PCR System (Thermo Fisher Scientific, Waltham, USA). Quantitative real-time PCR (qRT-PCR) was conducted with the following primer sequences: *Tyrosinase* forward, 5'-AGTCGTATCTGGCCATGGCTTCTTG-3' and reverse, 5'-GCAAGCTGTGGTAGTCGTCTTTGTC-3'; *Tyrp-1* forward, 5'-CTGCGATGTCTGCACTGATGACTTG-3' and reverse, 5'-TTTCTCCTGATTGGTCCACCCTCAG-3'; *Tyrp-2* forward, 5'- CGTGCTGAACAAGGAATGCT-3' and reverse, 5'-GCATGTCCGGTTGAAGAAT-3'; *Mitf* forward, 5'- AGAAGCTGGAGCATGCGAACC-3' and reverse, 5'-GTTCCTGGCTGCAGTTCTCAAG-3'; *Gapdh* forward, 5'-GTCTCCTCTGACTTCAACAGCG-3' and reverse, 5'-ACCACCCTGTTGCTGTAGCCAA-3'.

4.7. Melanin contents analysis using UVB-irradiated HaCaT keratinocytes-conditioned medium

The analysis of melanin contents in UVB-irradiated HaCaT keratinocytes-conditioned medium system was performed using the modified cell-based melanin biosynthesis method of previous reports (Chen et al. 2020; Hseu et al. 2020). HaCaT keratinocyte cells (1.0×10^6) were seeded for 24 h in a 37 °C incubator under the humidified atmosphere with 5% CO_2. After 24 h, the medium was removed and 1 ml of PBS was added for UVB irradiation. The cells were irradiated with 10 mJ/cm^2 of UVB using UV irradiator (UV-2AB, BoTeck, Gunpo, Korea) and 5 ml of media was added. Following the incubation for 24 h, the media was obtained from HaCaT cells and referred to as UVB- irradiated or UVB-nonirradiated conditioned medium. B16F10 melanoma cells were treated with the conditioned medium and nodakenin (10 and 25 μM) or arbutin for 24 h in a 37 °C incubator under the humidified atmosphere with 5% CO_2. After 48 h of incubation, the melanin content assay was performed using the same method as mentioned above.

4.8. Melanin contents analysis in UVB-irradiated HaCaT keratinocytes and B16F10 melanoma cells co-culture system

We analyzed the melanin contents in UVB-irradiated HaCaT keratinocytes and B16F10 melanoma cells co-culture system using the modified cell-based melanin biosynthesis method of previous report (Park et al. 2020). HaCaT cells (5×10^5) were cultured for 24 h and B16F10 cells (5×10^5) were added to HaCaT cells at a culturing ratio 5:5 for 24 h in a 37 °C incubator under the humidified atmosphere with 5% CO_2. Before UVB irradiation, the media was removed and 1 ml of PBS was added. Then, the cells were irradiated with UVB (10 mJ/cm^2) using UV irradiator (UV-2AB, BoTeck, Gunpo, Korea) and after UVB irradiation, PBS was removed and the media was added. The cells were incubated again in a 37 °C incubator under the humidified atmosphere with 5% CO_2. Following incubation for 48 h, the melanin contents were analyzed in the same way as mentioned above.

4.9. Statistical analysis

All data are expressed as mean±SD. Statistical analyses were performed with student's t-test or one-way ANOVA followed by Tukey's test. Significant difference was indicated at a value of $P < 0.05$ for all tests.

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