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Rosmarinic acid induces melanogenesis through protein kinase A activation signaling

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ABSTRACT

Melanogenesis is a physiological process that results in the synthesis of melanin pigments, which play a crucial protective role against skin photocarcinogenesis. In order to determine the effects of rosmarinic acid on melanogenesis and elucidate the molecular events of melanogenesis induced by rosmarinic acid, several experiments were performed in B16 melanoma cells. In this study, we showed that the melanin content and tyrosinase expression were increased by rosmarinic acid in a concentration-dependent manner. In addition, after the melanin content was increased by rosmarinic acid, it was reduced by H-89 and KT 5720, protein kinase A (PKA) inhibitors, but not by SB203580, a $p38^{mapk}$ inhibitor, or Ro-32-0432, a PKC inhibitor, which suggests the involvement of PKA in rosmarinic acid-induced melanogenesis. Consistent with this, rosmarinic acid induced the phosphorylation of CRE-binding protein (CREB), but had no effect on the phosphorylation of $p38^{mapk}$ or the inhibition of Akt phosphorylation. Additionally, rosmarinic acid induced the activation of cAMP response element (CRE) without having any effect on cAMP production, which suggests that rosmarinic acid-induced melanogenesis is mediated by PKA, which occurs downstream of cAMP production. This result was further confirmed by the fact that rosmarinic acid-induced phosphorylation of CREB was inhibited by H-89, but not by PD98059, a MEK1 inhibitor, or by LY294002, a phosphatidylinositol-3-kinase (PI3K) inhibitor. Rosmarinic acid-induced expression of tyrosinase protein was attenuated by H-89. Based on these results, we report for the first time that rosmarinic acid induces melanogenesis through PKA activation signaling.

1. Introduction

Skin pigmentation contributes significantly to the health and well-being of an individual. Pigment synthesized by cutaneous melanocytes protects the individual from various environmental assaults and potential cellular injury that can cause cancer and aging of the skin [1–3]. Within a keratinocyte, melanin/melanosome are preferentially localized over the nucleus [4]. In this position, melanin effectively absorbs ultraviolet light (UV) penetrating the skin and preventing consequential DNA damage. In addition, melanin is an effective scavenger of free radicals [5,6], further protecting

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Abbreviations: PKA, protein kinase A; CREB, CRE binding protein; GSK 3β, glycogen synthase kinase 3β; MITF, microphthalmia-associated transcription factor

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metabolically active keratinocytes from extensive environmental assaults. A lack of epidermal melanin increases susceptibility to skin cancers [1,2], and is an indicator of skin aging [7]. The loss of skin pigmentation can also result in compromised cutaneous immunity, resulting in conditions such as vitiligo [8–10]. Vitiligo is an acquired depigmentary disorder of the skin in which melanocytes of the skin are destroyed [11–13], resulting in melanotic lesions of variable size. Psychological and social problems related to self-esteem and personal interaction can be induced by vitiligo [14].

Melanin synthesis is stimulated by a large number of effectors, including 1-oleyl-2-acetyl-glycerol [15], ultraviolet-B radiation [16], cAMP-elevating agents (forskolin, IBMX, α-MSH, glycyrrhizin) [17–20], and placental total lipid fraction (PTLF) [21]. Thus far, three major important signaling pathways have been found to induce melanogenesis; one of these is the protein kinase C-mediated pathway, and the second is the cAMP-mediated pathway. While the role of protein kinase C in the induction of melanogenesis remains controversial, compelling data have shown that the cAMP pathway plays a key role in the regulation of melanogenesis, augmenting the enzyme activity of preexisting tyrosinase and increasing the amount of tyrosinase mRNA present [17–19]. Through the activation of PKA and CREB transcription factors, cAMP promotes an increase in the expression of microphthalmia-associated transcription factor (MITF) [22], a melanocyte-specific transcription factor that is crucial to the development and differentiation of melanocytes [23,24]. As a result, MITF binds to and activates the tyrosinase promoter, which leads to the stimulation of melanogenesis [25,26]. Finally, $p38^{mapk}$ signaling was recently found to be involved in melanogenesis [21,27]. The $p38^{mapk}$ cascade was more recently demonstrated to be involved in melanogenesis in B16 cells stimulated by treatment with α-MSH, UV light, or Lupeol [28–30].

Rosmarinic acid (α-o-caffeoyl-3,4-dihydroxyphenyl lactic acid) is a naturally occurring hydroxylated compound. It is widely distributed in the *Labitae* herbs, which include rosemary, sweet basil, and perilla [31–33]. Additionally, it has been reported that rosmarinic acid has the ability to block complement fixation, inhibit lipoxygenase and cyclooxygenase activities, and impede the Ca^{2+}-dependent pathways of TCR-mediated signaling by inhibiting PLC-γ1 and Itk activities [34–37]. Rosmarinic acid was recently found to inhibit the expression of CCL11 and CCR3 through the suppression of IKK β [38]. Because of these properties of rosmarinic acid, it has a broad range of applications, in products ranging from food preservatives and cosmetics, to medicine [39–41]. Although many reports on the anti-inflammatory and immunomodulatory effects of rosmarinic acid have been published, its effect on melanogenesis remains unknown. In our preliminary studies to screen pigmenting agents, we found that rosmarinic acid activates melanogenesis.

Therefore, using the well-characterized B16 mouse melanoma cells, we attempted to determine the effects and mechanisms of rosmarinic acid on melanogenesis. In this report, we demonstrated that rosmarinic acid induces melanogenesis through the activation of protein kinase A (PKA) without having a direct effect on cAMP production in B16 melanoma cells. We also found that rosmarinic acid could potentially be introduced as a possible therapeutic agent for disease of skin hypopigmentation such as vitiligo.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) and polyclonal rabbit antisera to human tyrosinase were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Antisera to phospho-$p38^{mapk}$, PD98059 (2′-amino-3′-methoxyflavone), antisera to phospho-Akt, antisera to phospho-CREB, and antisera to CREB were purchased from Cell Signaling Technology Inc. (Beverly, MA). SB203580 and LY294002 were purchased from Calbiochem (La Jolla, CA). Antibodies against β-actin, H-89, and forskolin were obtained from Sigma Chemical Co. Protease inhibitor cocktail was purchased from Roche (Indianapolis, IN). Chemiluminescence kits were purchased from Amersham Pharmacia Biotech (Buckinghamshire, England). CRE-Luc reporter plasmid was purchased from Stratagene.

2.2. Cell cultures

B16 melanoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and penicillin/streptomycin (100 IU/50 g/ml) in a humidified atmosphere containing 5% CO_2 in air at 37 °C.

2.3. MTT assay

The general viability of cultured cells was determined through the reduction of 3-(4,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to formazan. After rosmarinic acid treatment, cells were incubated for 48 h at 37 °C in a 5% CO_2 atmosphere. MTT (1 mg/ml in PBS) was added to each well at a 1/10 volume of media. Cells were incubated at 37 °C for 3 h, and dimethyl sulfoxide was added in order to dissolve the formazan crystals. The absorbance was then measured at 570 nm using a spectrophotometer.

2.4. Melanin content assay

Melanin contents of cultured B16 melanoma cells were measured according to the method of Oka et al. [42] with a slight modification. The colors of cell pellets were evaluated visually, and pellets were solubilized in boiling 1 M NaOH for 10 min. Spectrophotometric analysis of melanin content was performed at 400 nm absorbance.

2.5. Western blot analysis

B16 melanoma cell lysates were separated by SDS-PAGE (16% acrylamide gels) and transferred to Hybond-C membranes. The blots were probed with tyrosinase, β-actin, CREB, phospho-CREB, $p38^{mapk}$, and phospho-Akt antibodies. Proteins were then visualized using the Amersham ECL system.

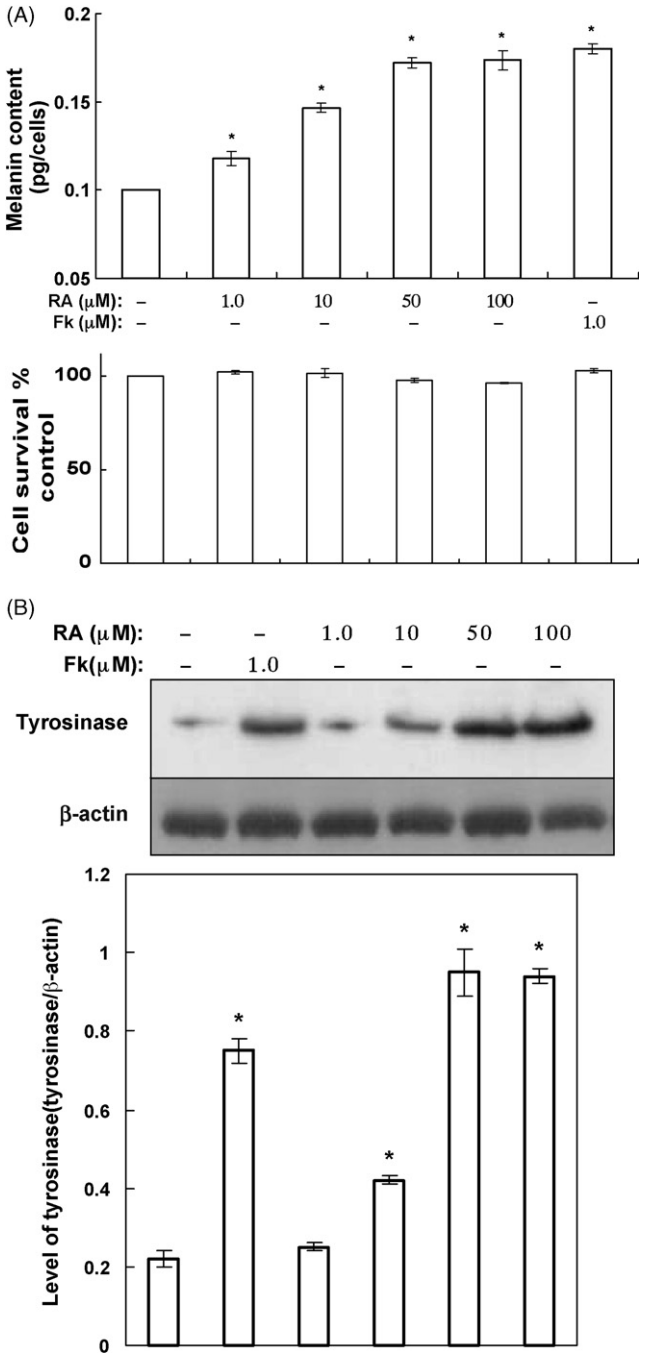

Fig. 1 – Rosmarinic acid increases both the melanin content and the expression of tyrosinase protein in B16 melanoma cells. (A) Melanin content assay and MTT assay, and (B) Western blot analysis of tyrosinase protein. B16 melanoma cells that had been treated with rosmarinic acid for 48 h were analyzed using a melanin content assay and an MTT assay, respectively (A). Results were confirmed by three independent experiments. Data are expressed as the percent change in melanin content compared to the untreated control (mean ± S.E.M.). *P < 0.05. Protein extracts from B16 melanoma cells that had been treated with rosmarinic acid for 48 h were subjected to Western blotting and probed, first with anti-tyrosinase antibodies, then with anti-β actin antibodies (B). Results were confirmed by three independent experiments. *P < 0.05, compared to the untreated control. Fk: forskolin; RA: rosmarinic acid.

2.6. Detection of phosphorylated $p38^{mapk}$

The phosphorylated MAP kinase isoform, $p38^{mapk}$, was identified by Western blot analysis using anti-phospho $p38^{mapk}$ antibody. In brief, cultured melanoma cells were washed with ice-cold phosphate-buffered saline (pH 7.4). Then, 0.2 ml of lysis buffer (10 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, 2 mM EGTA, 2 mM dithiothreitol, 1 mM sodium orthovanadate, 1 mM Phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin) was added, and B16 melanoma cells were subsequently harvested. After centrifugation at 16,000 × *g* for 30 min at 4 °C, the cell lysates were denatured in boiling water for 5 min. Samples of the supernatant were subjected to SDS-PAGE using a 16% polyacrylamide gel. After electrophoresis, proteins were transferred to a Hybond-C membrane at 4 °C. For the detection of phosphorylated $p38^{mapk}$, the sheets were immersed in blocking buffer containing 5% skim milk, 20 mM Tris-buffered saline (pH 7.4), and 0.1% Tween-20 for 3 h at 25 °C. Phosphorylation of $p38^{mapk}$ was detected by protein immunoblotting using a 1:1000 dilution of rabbit polyclonal dual phospho-specific antibodies. Blots were developed by enhanced chemiluminesence after incubation with HRP-conjugated secondary anti-rabbit IgG monoclonal antibody (1:2000 dilution) for 1 h at 25 °C, as recommended by the manufacturer.

2.7. Luciferase reporter assay

To assay for CRE promoter activity, B16 melanoma cells were transfected with CRE-Luc reporter along with Renilla luciferase expression vector driven by thymidine kinase promoter (Promega, WI) using Superfect™ reagent (Invitrogen, California). After incubation for 24 h, cells were stimulated for 14 h with rosmarinic acid, and were then harvested and lysed. Supernatants were assayed for luciferase activity. Luciferase activity was determined using a Dual Luciferase Assay system (Promega, WI) and a LB953 luminometer (Berthold, Germany), and was expressed as a ratio of the CRE-dependent firefly luciferase activity divided by the control thymidine kinase Renilla luciferase activity (% control). Results were confirmed by three independent transfections.

2.8. cAMP immunoassay

The cAMP concentration was analyzed using a cAMP kit from R&D Systems Inc. (Minneapolis, MN). In brief, B16 melanoma cells (7×10^4) were lysed in 0.1 M of HCl in order to inhibit phosphodiesterase activity. Supernatants were collected, neutralized, and diluted. Following neutralization and dilution, a fixed amount of cAMP conjugate (alkaline phosphate-labeled cAMP) was added to compete with cAMP in the cell lysates for sites on a rabbit polyclonal antibody immobilized on a 96-well plate. After one washing to remove excess conjugated and unbound cell lysate cAMP, a substrate solution was added to the wells to determine the bound enzyme activity. The color development was stopped, and the absorbance was then read at 405 nm. The intensity of the color is known to be inversely

proportional to the concentration of cAMP in the cell lysates. All studies were repeated at least three times. Data are expressed as means ± S.E.M. *$P < 0.05$ compared with untreated control.

2.9. Statistical evaluation

Means ± S.E.M. of the means were calculated; statistical analysis of results was performed using Student's *t*-test for independent samples. Values of *$P < 0.05$ were considered significant.

3. Results

3.1. Rosmarinic acid increases melanin content in B16 melanoma cells

As a first step to determining the effect of rosmarinic acid on melanogenesis, a melanin content assay and Western blot for tyrosinase were performed in B16 melanoma cells. As shown in Fig. 1A, rosmarinic acid increases melanin content, since melanin formation was found to be enhanced in response to serial concentrations of rosmarinic acid (1, 10, 50, and 100 μM, respectively). To exclude the possibility that upsurges in melanin content may have been induced by the cell-proliferating effect of rosmarinic acid, the MTT assay was performed (Fig. 1A). Expression of tyrosinase protein was also found to be increased by rosmarinic acid in a concentration-dependent manner (Fig. 1B). Forskolin was employed as a positive control.

3.2. Rosmarinic acid-induced increase of melanin content was inhibited by H-89 and KT 5720, protein kinase A inhibitors

Several molecules have been reported to induce melanogenesis, namely $p38^{mapk}$, PI3K, and protein kinase A (PKA). In order to elucidate the mechanisms of rosmarinic acid-induced

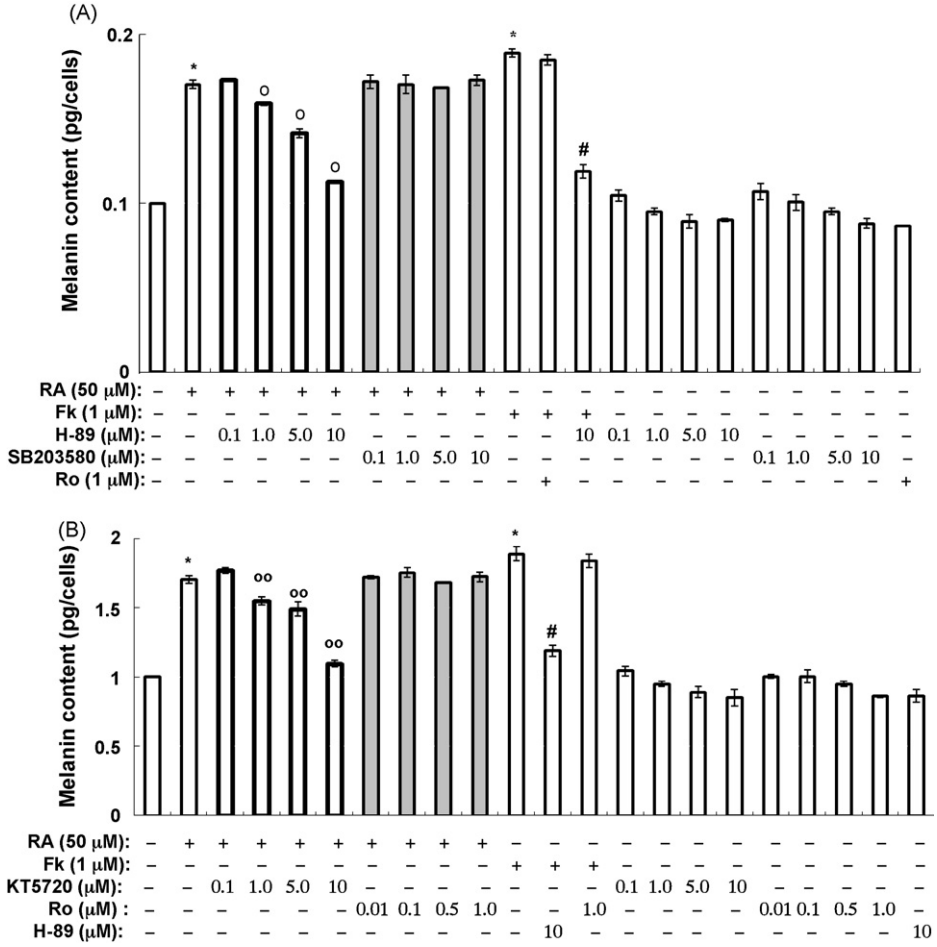

Fig. 2 – The rosmarinic acid-induced increase of melanin content was reduced by H-89 and KT 5729, protein kinase inhibitors, but not by SB203580, a $p38^{mapk}$ inhibitor, or Ro-32-0432, a PKC inhibitor. B16 melanoma cells that had been pretreated with the indicated concentrations of H-89 and SB203580 (A) or KT 5720 and Ro-32-0432 (B) for 1 h were then treated for 48 h with rosmarinic acid and analyzed using a melanin content assay. Results were confirmed by three independent experiments. Data are expressed as the percent change in melanin content compared to the untreated control (mean ± S.E.M.). Data are expressed as indicated in the Section 2. *$P < 0.05$, compared to the untreated control, °$P < 0.05$ vs. rosmarinic acid (50 μM) only, #$P < 0.05$ vs. forskolin (1 μM) only, °°$P < 0.05$ vs. KT 5720. Fk: forskolin; RA: rosmarinic acid; Ro: Ro-32-0432.

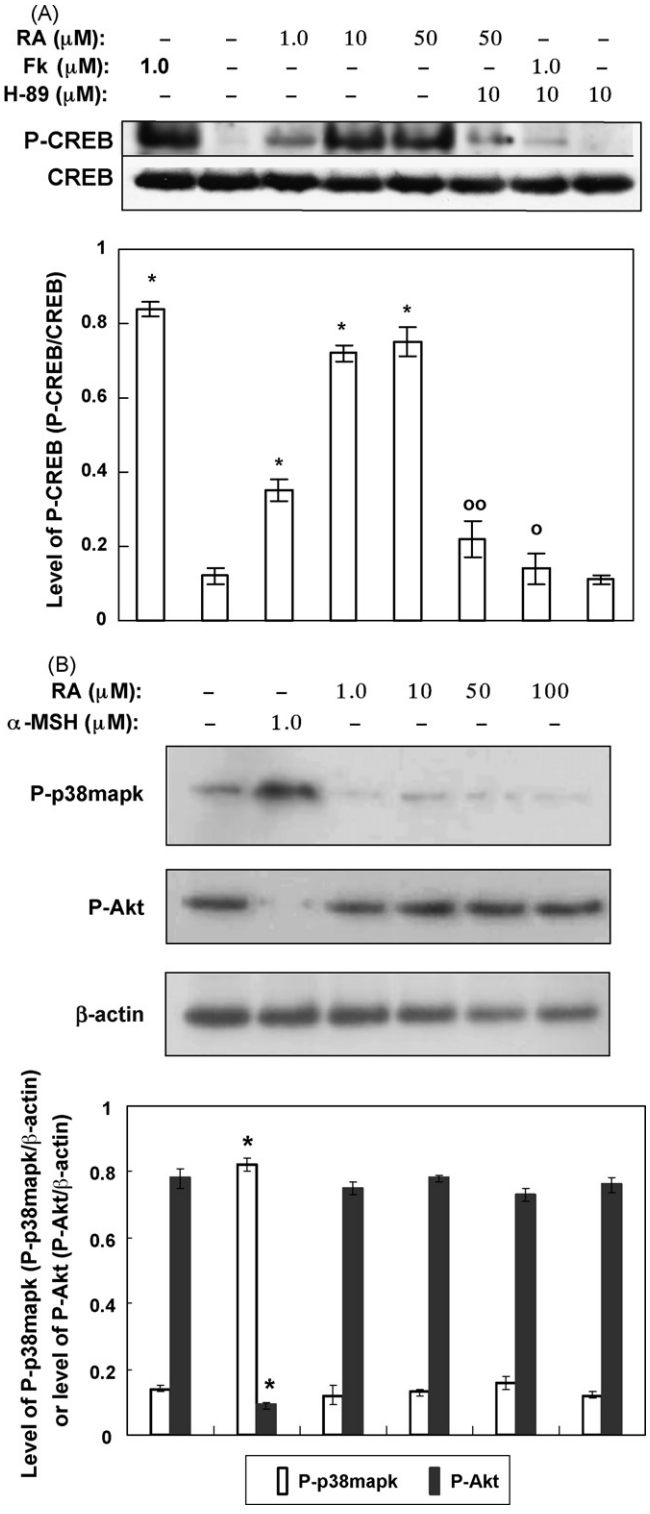

Fig. 3 – Rosmarinic acid induces the phosphorylation of CREB via PKA (A), but has no effect on the phosphorylation of p38^{mapk} or the inhibition of Akt phosphorylation (B). B16 melanoma cells were left untreated or were incubated with the indicated concentrations of rosmarinic acid or 10 μM forskolin for 15 min. Additional B16 melanoma cells pretreated with or without 10 μM H-89 for 1 h were incubated with 50 μM rosmarinic acid for 15 min (A) or 1 μM α-MSH and the indicated concentrations of rosmarinic acid for 2 h (B). Western blotting was then performed with phospho-specific antibodies such as phospho-CREB, phospho-p38^{mapk}, and phosphor-Akt antibody. Detection of CREB and β-actin showed that each lane was loaded with equal amounts of protein. Results were confirmed by three independent experiments, respectively. $^{*}P < 0.05$, compared to the untreated control, $^{o}P < 0.05$ vs. forskolin (1 μM) only, $^{oo}P < 0.05$ vs. forskolin (1 μM) only, $^{\#}P < 0.05$ vs. α-MSH (50 μM) only. Fk: forskolin; RA: rosmarinic acid.

melanogenesis, we utilized SB203580, a p38^{mapk} inhibitor, Ro-32-0432, a PKC inhibitor, and KT 5720 and H-89 as PKA inhibitors to perform a melanin content assay. As shown in Fig. 2, while SB203580 and Ro-32-0432 did not inhibit rosmarinic acid-induced melanogenesis, H-89 and KT 5720 suppressed rosmarinic acid-induced melanin formation. This result suggests that PKA may be involved in rosmarinic acid-induced melanogenesis. As a positive and negative control, H-89 plus forskolin and Ro-32-0432 plus forskolin, respectively, were employed.

3.3. *Rosmarinic acid induces phosphorylation of CREB through PKA, but has no effect on p38^{mapk} phosphorylation or inhibition of Akt phosphorylation*

Fig. 2 shows the possibility that rosmarinic acid has the potential to induce the activation of PKA, and also suggests that the effect of the activation of PKA by rosmarinic acid may contribute to the activation of the MITF promoter, since its promoter has a CRE element, and may thereby induce melanogenesis.

To further confirm this finding, we designed an experiment to elucidate whether rosmarinic acid can induce the phosphorylation of CREB and p38^{mapk}, or whether it is capable of stimulating a reduction of Akt phosphorylation in B16 melanoma cells, as these events are known to be involved in melanogenesis [43]. As shown in Fig. 3 A, we discovered that rosmarinic acid activated CREB, in accordance with the H-89-mediated inhibition of a rosmarinic acid-induced increase of melanin content. While CREB phosphorylation was attenuated by H-89 (Fig. 3A), PD98059, a MEK1 inhibitor, and LY294002, a PI3K inhibitor, did not suppress rosmarinic acid-induced CREB phosphorylation (data not shown). In addition, rosmarinic acid did not induce p38^{mapk} phosphorylation, nor did it stimulate the inhibition of Akt phosphorylation (Fig. 3B). GSK 3β phosphorylation also was not inhibited (data not shown). These results strengthen the possibility that rosmarinic acid may induce melanogenesis through the activation of CREB by PKA. Forskolin and α-MSH treatment were employed as positive controls.

3.4. *Rosmarinic acid activates protein kinase A independently of camp*

In our study, we found that rosmarinic acid activates protein kinase A. In order to determine whether or not rosmarinic acid is involved in the production of cAMP, which activates protein kinase A, we performed CRE reporter and cAMP production assays. As shown in Fig. 4A, rosmarinic acid activated CRE

reporter activity, and its CRE-activating effect was inhibited by H-89, a PKA inhibitor, thus confirming that rosmarinic acid operates upstream of PKA. However, cAMP production was not induced by rosmarinic acid (Fig. 4B and C). These results suggest that rosmarinic acid activates PKA independently of cAMP. Forskolin was employed as a positive control.

3.5. *Rosmarinic acid regulates tyrosinase expression through the activation of PKA*

To directly demonstrate the involvement of PKA in rosmarinic acid-induced melanogenesis, a Western blot for tyrosinase was performed using H-89, a PKA inhibitor. As shown in Fig. 5, the rosmarinic acid-induced upregulation of tyrosinase expression was reduced by H-89. Therefore, these results indicate the involvement of PKA in rosmarinic acid-induced melanogenesis.

4. Discussion

In this report, we investigated the pigmenting effect and the molecular mechanism by which rosmarinic acid elicits its activating effects on melanogenesis. We demonstrated that rosmarinic acid upregulates tyrosinase expression, which strongly suggests that rosmarinic acid increases melanogenesis through the activation of tyrosinase expression. Then, among the different pathways that are known to be involved in melanogenesis, including the $p38^{mapk}$, PI3K, and PKA (protein kinase A) pathways, we found that rosmarinic acid induces melanogenesis by activating the PKA pathway.

Although rosmarinic acid (α-o-caffeoyl-3, 4-dihydroxyphenyl lactic acid) has been found to have anti-inflammatory and immunomodulatory functions, until now, the effects of rosmarinic acid on pigmentation have not been investigated. Therefore, in this study, we attempted to investigate the

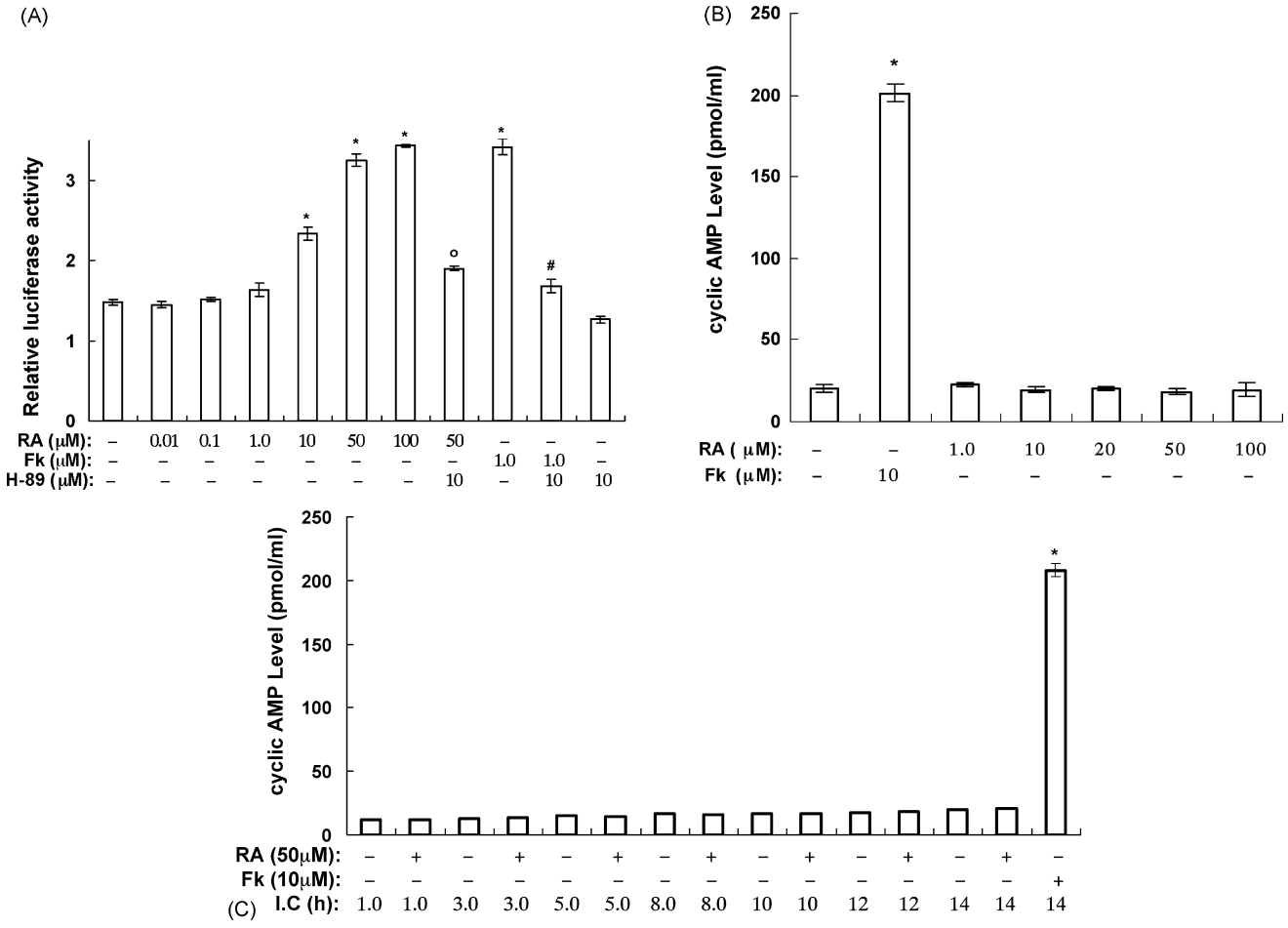

Fig. 4 – Rosmarinic acid induces the activation of CRE via PKA, and has no effect on cAMP production in B16 melanoma cells. B16 melanoma cells were transfected with CRE-Luc reporters along with a Renilla luciferase expression vector driven by a thymidine kinase promoter (Promega) using Superfect™ reagent (Invitrogen). After incubation for 24 h, cells pretreated with or without 10 μM H-89 for 1 h were stimulated with rosmarinic acid for 14 h. These cells were then harvested, lysed, and assayed. Results were confirmed by three independent transfections. Data are expressed as means ± S.E.M. *P < 0.05 compared to the untreated control. °P < 0.05 vs. rosmarinic acid only. #P < 0.05 vs. forskolin only (A). B16 melanoma cells were treated with the indicated concentrations of rosmarinic acid or forskolin (10 μM) (B) for 14 h or with rosmarinic acid (50 μM) for a serial period of incubation (1, 3, 5, 8, 10, or 14 h) (C). After treatment, the cAMP level was detected using a cAMP kit from R&D Systems Inc. Results were confirmed by three independent experiments. Data are expressed as means ± S.E.M. *P < 0.05 compared to the untreated control. Fk: forskolin; RA: rosmarinic acid; I.C.: incubation time.

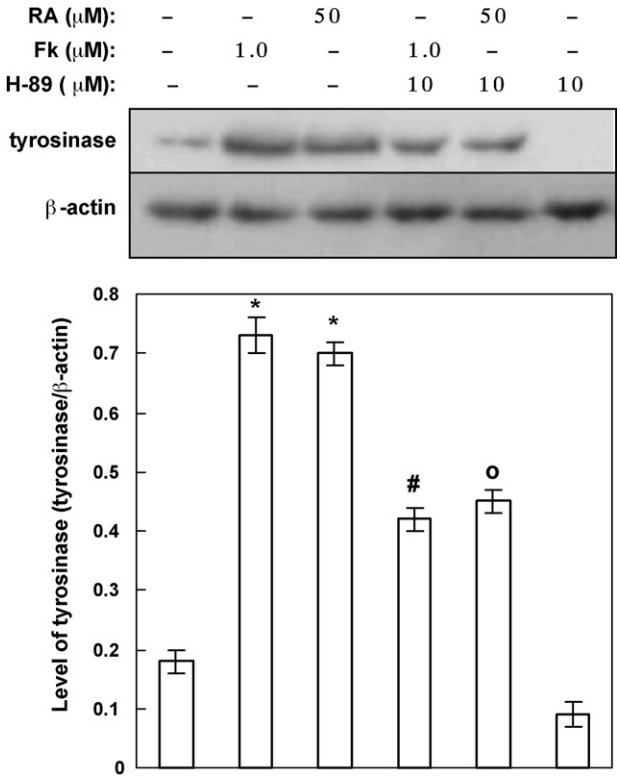

Fig. 5 – Effect of H-89 on the rosmarinic acid-induced increase of tyrosinase expression. B16 melanoma cells that had been pretreated with or without 10 μM H-89 for 1 h were stimulated with rosmarinic acid (50 μM) or forskolin (10 μM) for 48 h, and were then subjected to Western blotting using anti-tyrosinase Ab, an anti-β actin Ab. Results were confirmed by three independent experiments. Data are expressed as the percent change in melanin content compared to the untreated control (mean ± S.E.M.). $^{*}P < 0.05$ compared to the untreated control, $^{o}P < 0.05$ vs. rosmarinic acid (50 μM) only, $^{\#}P < 0.05$ vs. forskolin only. Fk: forskolin; RA: rosmarinic acid.

possibility that rosmarinic acid may exhibit a pigmenting or depigmenting effect in B16 melanoma cells. In the results of this investigation, we found that rosmarinic acid increases the melanin content and the expression of tyrosinase protein in these cells. In order to determine the mechanisms of action of the pigmenting effect of rosmarinic acid, melanin content assays were performed using SB203580, a $p38^{mapk}$ inhibitor, and H-89, a PKA inhibitor. In this study, we found that while SB203580 has no effect on rosmarinic acid-induced melanogenesis, rosmarinic acid-induced melanin formation is suppressed by H-89. These findings suggest that PKA may be involved in rosmarinic acid-induced melanogenesis.

PKA has been reported to be involved in cAMP-induced melanogenesis through the activation of CREB phosphorylation, which consequently induces melanogenesis. Our previous results also suggest that rosmarinic acid may induce melanogenesis through the activation of PKA. Therefore, we investigated the effect of rosmarinic acid on the phosphorylation of CREB. In this study, the phosphorylation of CREB was induced by rosmarinic acid in a concentration-dependent manner. To further confirm this fact, we designed an experiment to elucidate whether rosmarinic acid can also induce the phosphorylation of $p38^{mapk}$ or stimulate a reduction of Akt phosphorylation in B16 melanoma cells because these events are well known to be involved in melanogenesis. As expected, rosmarinic acid did not induce $p38^{mapk}$ phosphorylation, nor did it inhibit the phosphorylation of Akt. These results strengthen the possibility that rosmarinic acid induces melanogenesis through the phosphorylation of CREB by PKA.

Intracellular signaling pathways such as the phosphatidylinositol 3-kinase (PI3K), extracellular signal-regulated kinase (ERK), and protein kinase A (PKA) pathway have been shown to be involved in CREB phosphorylation [44–48]. These reports prompted us to investigate the specific kinases that are involved in the phosphorylation of CREB by rosmarinic acid. The rosmarinic acid-induced phosphorylation of CREB was found to be attenuated by H-89, but not by PD98059, an MEK1 inhibitor, or by LY294002, a PI3K inhibitor. This result indicates the involvement of PKA in rosmarinic acid-induced melanogenesis.

Until now, it was believed that rosmarinic acid induces melanogenesis through the activation of PKA. In general, cAMP-elevating agents, including forskolin, IBMX, α-MSH, and glycyrrhizin, are known to induce melanogenesis through the activation of PKA [17–19]. Therefore, we investigated whether or not rosmarinic acid induces cAMP production by using a CRE promoter reporter assay and EIA for cAMP. The assays indicated that although rosmarinic acid activates CRE reporter activity, cAMP production is not induced by rosmarinic acid (Fig. 4), and these results suggest that rosmarinic acid activates protein kinase A in a cAMP-independent manner. Interactions of TGF β pathway components with effectors of other signaling pathways have recently been described. One potentially important interaction was suggested by a report which indicated that TGF β could activate PKA through an unknown mechanism associated with the Smad3/Smad4 complex [49]. In addition, TNF α-induced reactive oxygen species were reported to induce the activation of PKA [50]. Therefore, it is possible that rosmarinic acid may activate PKA through interactions with other cellular signaling pathways such as those of TGF β and TNF α. However, the possibility that the activation of PKA by rosmarinic acid may be mediated by a cAMP-dependent pathway cannot be completely excluded. In order to elucidate this, further study will be required.

Many current investigations have focused on the specific mechanisms involved in melanogenesis in order to develop new therapeutic agents for skin pigmentation abnormalities. Along these lines, the agents that stimulate melanin production can also be used as skin tanning agents. Dihydroxyacetone-containing preparations have been used to induce temporary pigmentation that resembles a UV-induced tan [51,52]. The tan produced by UVB and/or UVA radiation is photoprotective against subsequent UV exposure. DHA also produces cosmetically acceptable pigmentation of vitiliginous skin [53]. Therefore, utilization of the pigmenting effect of rosmarinic acid might be useful as an adjunctive therapy for treatment of hypopigmentation-related disorders, as well as for tanning.

Taken together, the data gathered in this study demonstrate that rosmarinic acid induces melanogenesis by activating the CREB via PKA. Additionally, the fact that rosmarinic acid activated CRE promoter reporter activity and CREB phosphorylation but did not induce the phosphorylation of $p38^{mapk}$ or the inhibition of Akt phosphorylation suggests that rosmarinic acid operates downstream of cAMP production or in a cAMP-independent manner.

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