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Research Article

Phenolic acids in *Panax ginseng* inhibit melanin production through bidirectional regulation of melanin synthase transcription via different signaling pathways

Jianzeng Liu [a, 1], Xiaohao Xu [a, b, c, 1], Jingyuan Zhou [b, 1], Guang Sun [b], Zhenzhuo Li [b], Lu Zhai [b], Jing Wang [b], Rui Ma [b], Daqing Zhao [a], Rui Jiang [b, d, *], Liwei Sun [b, d, **]

[a] *Northeast Asian Institute of Traditional Chinese Medicine, Changchun University of Chinese Medicine, Changchun, China*
[b] *Research Center of Traditional Chinese Medicine, the Affiliated Hospital to Changchun University of Chinese Medicine, Changchun, China*
[c] *Jilin Ginseng Academy, Changchun University of Chinese Medicine, Changchun, China*
[d] *Key Laboratory of Active Substances and Biological Mechanisms of Ginseng Efficacy, Ministry of Education, Changchun, China*

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A B S T R A C T

Background: Our previous investigation indicated that the preparation of *Panax ginseng* Meyer (*P. ginseng*) inhibited melanogenesis. It comprised salicylic acid (SA), protocatechuic acid (PA), *p*-coumaric acid (*p*-CA), vanillic acid (VA), and caffeic acid (CA). In this investigation, the regulatory effects of *P. ginseng* phenolic acid monomers on melanin production were assessed.
Methods: *In vitro and in vivo* impact of phenolic acid monomers were assessed.
Results: SA, PA, *p*-CA and VA inhibited tyrosinase (TYR) to reduce melanin production, whereas CA had the opposite effects. SA, PA, *p*-CA and VA significantly downregulated the melanocortin 1 receptor (MC1R), cycle AMP (cAMP), protein kinase A (PKA), cycle AMP-response element-binding protein (CREB), microphthalmia-associated transcription factor (MITF) pathway, reducing mRNA and protein levels of TYR, tyrosinase-related protein 1 (TYRP1), and TYRP2. Moreover, CA treatment enhanced the cAMP, PKA, and CREB pathways to promote MITF mRNA level and phosphorylation. It also alleviated MITF protein level in α-MSH-stimulated B16F10 cells, comparable to untreated B16F10, increasing the expression of phosphorylation glycogen synthase kinase 3β (p-GSK3β), β-catenin, p-ERK/ERK, and p-p38/p38. Furthermore, the GSK3β inhibitor promoted p-GSK3β and p-MITF expression, as observed in CA-treated cells. Moreover, p38 and ERK inhibitors inhibited CA-stimulated p-p38/p38, p-ERK/ERK, and p-MITF increase, which had negative binding energies with MC1R, as depicted by molecular docking.
Conclusion: *P. ginseng* roots' phenolic acid monomers can safely inhibit melanin production by bidirectionally regulating melanin synthase transcription. Furthermore, they reduced MITF expression via MC1R/cAMP/PKA signaling pathway and enhanced MITF post-translational modification via Wnt/mitogen-activated protein kinase signaling pathway.
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1. Introduction

Excessive melanin production causes skin hyperpigmentation (darkening of the skin), in severe cases, develops medically serious dermatological conditions such as melasma, freckles, and post-inflammatory hyperpigmentation [1]. Melanin is mainly synthesized from tyrosine by multiple reactions catalyzed by key enzymes, including tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1) and (TYRP2) [2]. Importantly, skin pigmentation can be reduced by decolorizing agents that inhibit enzymes associated with melanin synthesis from tyrosine [3]. However, melanin can

* Corresponding author. Research Center of Traditional Chinese Medicine, the Affiliated Hospital to Changchun University of Chinese Medicine, 1478 Gongnong Street, Changchun, Jilin Province, 130021, China.
** Corresponding author. Research Center of Traditional Chinese Medicine, the Affiliated Hospital to Changchun University of Chinese Medicine, 1478 Gongnong Street, Changchun, Jilin Province, 130021, China.
E-mail addresses: jiangrui800710@163.com (R. Jiang), sunnylilwei@163.com (L. Sun).
[1] These authors contributed equally to this work.

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also protect epidermal keratinocytes from the damaging effects of ultraviolet (UV) radiation, as excessive melanin synthesis inhibition causes irreversible skin damage [4,5]. Therefore, researchers are working to develop safer and more effective topical skin treatments comprising decolorizing agents that reduce excessive melanin production and UV-absorbing agents that prevent UV exposure-induced skin damage [6].

Researchers in various fields have identified the beneficial effects of natural products in alleviating numerous human disorders [7–11]. Many investigations have been carried out to identify melanogenesis-suppressing natural products. Multiple plant-based melanogenesis inhibitors have been discovered, including terpenes, phenolics, long-chain fatty acids, steroids, flavonoids, chalcones, alkaloids, and coumarins, whereas some others, including hydroquinone, arbutin, and aloin, are currently being used as skin-whitening agents [12]. Their mechanisms include TYR inhibition, MITF expression downregulation, and modulation of related signaling pathways [13–16]. Although many natural products have an inhibitory effect on pigmentation, the main problems with these inhibitors are their toxicity and high frequency of adverse reactions [17–19]. Therefore, safe and more efficient therapeutic options are increasingly needed. Consequently, various active ingredients have been discovered that modulates human health by regulating bidirectional regulatory pathway [20–22]. For example, ginsenoside Rg3 and F1 inhibit melanin synthesis in normal human epidermal melanocytes and B16F10 cells, while ginsenosides Rb1 and Rg1 promote melanogenesis and TYR in human melanocytes [23–25]. These results suggest that *Panax ginseng* Meyer *(P. ginseng)* may contain active components inhibiting and promoting melanin synthesis-related processes. We previously reported the phenolic acids presented in *P. ginseng* roots, such as salicylic acid (SA), protocatechuic acid (PA), *p*-coumaric acid (*p*-CA), vanillic acid (VA) and caffeic acid (CA) [26]. Although SA and VA have been reported to inhibit melanin production [27,28], it is unclear whether these compounds collectively exert a two-way regulatory effect involving concurrent inhibition and stimulation of melanin production. This two-way mechanism would alleviate, but not eliminate, skin melanin production and prevent hyperpigmentation while also protecting the skin from UV damage.

Microphthalmia-associated transcription factor (MITF) upregulates TYR expression and is a major stimulating of melanin synthesis [29,30]. Melanin production is induced by α-melanocyte stimulating hormone (α-MSH), released from keratinocytes in response to UV irradiation, and binds with melanocortin 1 receptor (MC1R) to activate the cyclic AMP/protein kinase A (cAMP/PKA) signaling pathway, thereby, stimulates the phosphorylation of cAMP-response element-binding protein (CREB), and initiate MITF transcription [31]. Importantly, MITF transcriptional activity is influenced by MITF protein posttranslational modifications, including phosphorylation [15]. Once MITF is phosphorylated, the resulting p-MITF enters the nucleus to promote the transcription of TYR, controlled by phosphorylation-specific amino acid residues within ERK1/2, glycogen synthase kinase 3β (GSK3β), and p38 [15,32–35]. Therefore, melanin synthesis is influenced by both MITF mRNA expression levels and by MITF-associated post-transcriptional phosphorylation status.

This investigation elucidated the impacts and the mechanisms of action of five phenolic acid monomers isolated from *P. ginseng* roots on melanogenesis in melanocytes and the zebrafish model. It was revealed that a combination of different *P. ginseng* root phenolic acid compounds can safely inhibit melanin production via bidirectional regulation through different pathways, suggesting that *P. ginseng* root active substances have anti-melanogenic effects.

2. Materials and methods

2.1. Chemicals and reagents

The chemicals utilized, SA, PA, p-CA, VA and CA (purity≥98) were purchased from the National Institutes for Food and Drug Control (Beijing, China), and their catalog number were 100106, 110809, 112037, 110776 and 110885, respectively. Monoclonal antibodies against TYR (Cat# ab170905), TYRP2 (Cat# ab221144), MITF (Cat# ab140606), TYRP1 (Cat# ab235447), and p-MITF (Cat# ab59201) (Abcam, Cambridge, MA, USA). Monoclonal antibodies against GAPDH (Cat# 5174), p-CREB (Cat# 9198)/CREB (Cat# 9197), p-GSK3β (Cat# 9326), p-p38 (Cat# 4511) /p38 (Cat# 8690), p-ERK (Cat# 4370)/ERK (Cat# 4696) and β-catenin (Cat# 8480) (Cell Signaling Technology Inc., Beverly, MA, USA). α-MSH, L-dihydroxyphenylalanine (L-DOPA), and arbutin (Sigma-Aldrich, St. Louis, MO, USA).

2.2. P. ginseng phenolic acids extract and constituent monomers preparation

In September 2012, a 5-year-old *P. ginseng* root was acquired from Fusong in Jilin Province, China, as confirmed by Jie Wu, a *P. ginseng* expert from Jilin Province. The voucher specimen (2068) was submitted to the Herbarium of the College of Chemistry and Biology, Beihua University, Jilin Province, China. *P. ginseng* phenolic acids extract was prepared using a previously reported method [26]. Briefly, *P. ginseng* roots were sliced into small pieces and extracted with water; then, the aqueous extract was concentrated and extracted with ethyl acetate. The extract comprised five phenolic acid monomeric compounds SA, PA, *p*-CA, VA, and CA as identified via HPLC and LC-MS, in concentrations 0.31 ± 0.02 g/100 g (3.1 μg/mL), 0.42 ± 0.02 g/100 g (4.2 μg/mL), 0.12 ± 0.01 g/100 g (1.2 μg/mL), 6.85 ± 0.34 g/100 g (68.5 μg/mL), and 0.14 ± 0.01 g/100 g (1.4 μg/mL), respectively. These concentrations were subsequently used to assess each phenolic compound's melanin synthesis inhibiting capability. The 1 mg/mL of phenolic acid extract markedly inhibited melanin production.

2.3. Cell cultures

Murine melanoma cells (B16F10) (Cell Resource Center of the Shanghai Institute for Biological Sciences, Shanghai, China) were propagated in Dulbecco's modified Eagle's medium (DMEM, Gibco) augmented with 10% foetal bovine serum (FBS, Gibco, MA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin in 5% CO_2, humidified atmosphere, and at 37°C.

2.4. Melanin content identification

The melanin levels of B16F10 cells were assessed by optimizing the protocol from the previous investigation [28]. The cells were incubated with SA (3.1 μg/mL), PA (4.2 μg/mL), p-CA (1.2 μg/mL), VA (68.5 μg/mL) or CA (1.4 μg/mL) with arbutin (1 mM) and/or α-MSH (0.3 μM) for 48 h, lysed at 80°C using 100 μL of 1 N NaOH (containing 10% DMSO) for 2 h, transferred to 96-well plates, and then their melanin levels were quantified via a microplate reader at of 405 nm.

2.5. Tyrosinase activity assessment

The activity of TYR was estimated by calculating the rate of L-DOPA oxidation, as indicated reported [28]. At 4°C, RIPA lysis buffer

(Beyotime Biotechnology, Shanghai, China) was used to lyse B16F10 cells for 1 h, the supernatants (80 μg of protein) and L-DOPA (5 mM, 80 μL) were inoculated in 96-well microplates for 30 min at 37°C, and the dopachrome formation was measured at 492 nm via microplate reader.

2.6. Enzyme-linked immunosorbent assay (ELISA)

Supernatants acquired from the aforementioned protocols were utilized to elucidate the activities of adenylyl cyclase (AC) and cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) and cAMP levels in B16F10 cells with the help of ELISA assay kits (Boster Biological Technology, Wuhan, China) as per the manufacturer's guide. Data were depicted as a percentage of the α-MSH-induced group value.

2.7. RNA isolation and qRT-PCR

Briefly, whole cellular RNA was isolated after various treatment using Trizol reagent (Ambion, Austin, TX, USA). 5 μg of this RNA was templated to synthesise cDNA with Maxima First Strand cDNA Synthesis Kit (Thermo Scientific). Thereafter, mRNA levels corresponding to the transcription genes encoding MITF, TYR, TYRP1 and TYRP2 proteins were assessed using Luna Universal qPCR Master Mix (New England Biolabs, Ipswich, MA, USA) as per manufacturer's protocol, with PCR amplification conducted using a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). PCR primers are listed as follow: MITF, 5′-GTGAATCGGATCATCAAGCAAG-3' (F) and 5′-GAGATCCAGAGTTGTCGTACA T-3' (R); TYR, 5′-TTTCAACTGCGGAAACTGTAAG-3’ (F) and 5′-ATTGTTCATTTG GCCA TAGGTG-3' (R); TYRP1, 5′-GATCCAGAAGCAACTTCGATTC-3' (F) and 5′-TACAAAGTGTTCCCAGGGTATC-3' (R); TYRP2, 5′-GAGTATCCATCCAGACTA CGTG-3' (F) and 5′-CGTCCTGGACCTAATAATGTGT-3' (R); β-actin, 5′-CTACCTC ATGAAGATCCTGACC-3' (F) and 5′-CACAG CTTCTCTTTGATGTCAC-3' (R). Target genes data were normalized to β-actin, while their expression levels were normalized to control levels. The experiment was repeated thrice for validation.

2.8. Western blot analysis

The cell lysate was acquired as mentioned in section 2.5, and the proteins were isolated on gradient SDS-PAGE gels (8% or 12%) by electrophoresis, transported onto PVDF membranes, blocked with Tris-buffered saline plus 0.05% Tween-20 (TBST) augmented with 5% skimmed milk for 1 h, incubated overnight in primary antibodies at 4°C, rinsed with TBST, labeled with HRP-linked secondary antibodies, and lastly, the blots were visualized using an enhanced chemiluminescence reagent (Millipore Corporation, Billerica, USA).

2.9. Zebrafish husbandry

Zebrafish were used to assess the depigmenting effects of P. ginseng phenolic acids in vivo as previously reported, with several modifications [28,36]. Adult AB zebrafish were housed in maintained in polystyrene aquarium 3 L tanks (15 zebrafish per tank) and were provided standard 28.5°C with a 14 h light/10 h dark cycle. For acquiring embryos, natural mating was allowed by housing males and females (in a ratio of two males to one female) in a 1.0 L breeding tank. All experiments were performed following the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Ethics Committee of Changchun University of Chinese Medicine (Approval No. 2020195).

2.10. In-vivo anti-melanogenic analysis

Melanin and TYR activities were quantified after optimizing previously reported protocols [37]. 6-8 h post-fertilization embryos of the experimental set were incubated with different SA, PA, p-CA, VA and CA concentrations, and those of the positive control group were exposed to phenylthiourea (PTU) for 72 h. After incubation, the embryos were anaesthetized in tricaine methanesulphonate solution (0.02%) and visualized by stereomicroscope (Nikon SMZ745T, Tokyo, Japan). Melanin contents and TYR activities within embryo were detected using the abovementioned methods.

2.11. Molecular docking simulation

Molecular Operating Environment (MOE) v2018.01011 was utilized for molecular docking. 3D structures of MC1R protein were built using AlphaFold. For ligand modeling, PubChem imported 2D structures of molecules were converted to 3D in MOE, according to energy minimization. Before docking, the force field of AMBER10: EHT and Reaction Field (R-field) implicit solvation model were selected. The native ligands' binding site within the protein structure was selected as the binding pocket for ligand compounds.

The “induced fit” method was opted, where the receptor proteins binding sites' side chains were permitted to move according to ligand conformations within constraint limits to limit their positions. The weight utilized for tethering side chain atoms to their original positions was 10. First, the London dG scoring function for the docking simulation was utilized to rank the docked poses. Then, on the top 30 poses, a force field refinement was applied, then a rescoring of GBVI/WSA dG scores was performed using a scoring function. The lowest binding free energy value conformation was identified as the best probable binding mode [38].

2.12. Statistical analysis

The results are depicted as the mean ± SD from three separate experiments. Statistical measurement was conducted by one-way ANOVA, Tukey's post hoc test on GraphPad Prism 9 project (GraphPad Software, San Diego, CA, USA). p-values <0.05 were deemed statistically important.

3. Results and discussion

3.1. SA, PA, p-CA and VA reduced melanin synthesis by inhibiting TYR in α-MSH-treated B16F10 cells, while CA had the opposite effect

As Fig. 1A depicts, intracellular melanin levels and TYR activity of α-MSH-induced B16F10 cells were substantially increased than the control unstimulated cells [39]. However, SA, PA, *p*-CA, and VA treatment alleviated melanin and TYR activity in α-MSH-stimulated cells; similar inhibition was observed in arbutin-treated cells (the positive control). However, when the effects of SA, PA, *p*-CA, and VA were compared with CA treatment, opposite effects were observed; melanin content and TYR activity were further increased in CA-treated α-MSH-triggered cells than in untreated, stimulated cells. From the abovementioned results, SA, PA, *p*-CA, and VA treatment inhibited TYR activity and melanin production, while CA treatment promoted intracellular TYR activity and enhanced melanin production. We had previously reported that *P. ginseng* phenolic acids preparation comprised PA, VA, *p*-CA, SA, and CA and inhibited melanogenesis [26]. Although SA and VA have been reported to inhibit melanin production [27,28], the literature on the impact of CA on melanogenesis is controversial [40,41]. The regulatory impact of *P. ginseng* phenolic acid monomers on melanin production still needs further research. Indicating that phenolic

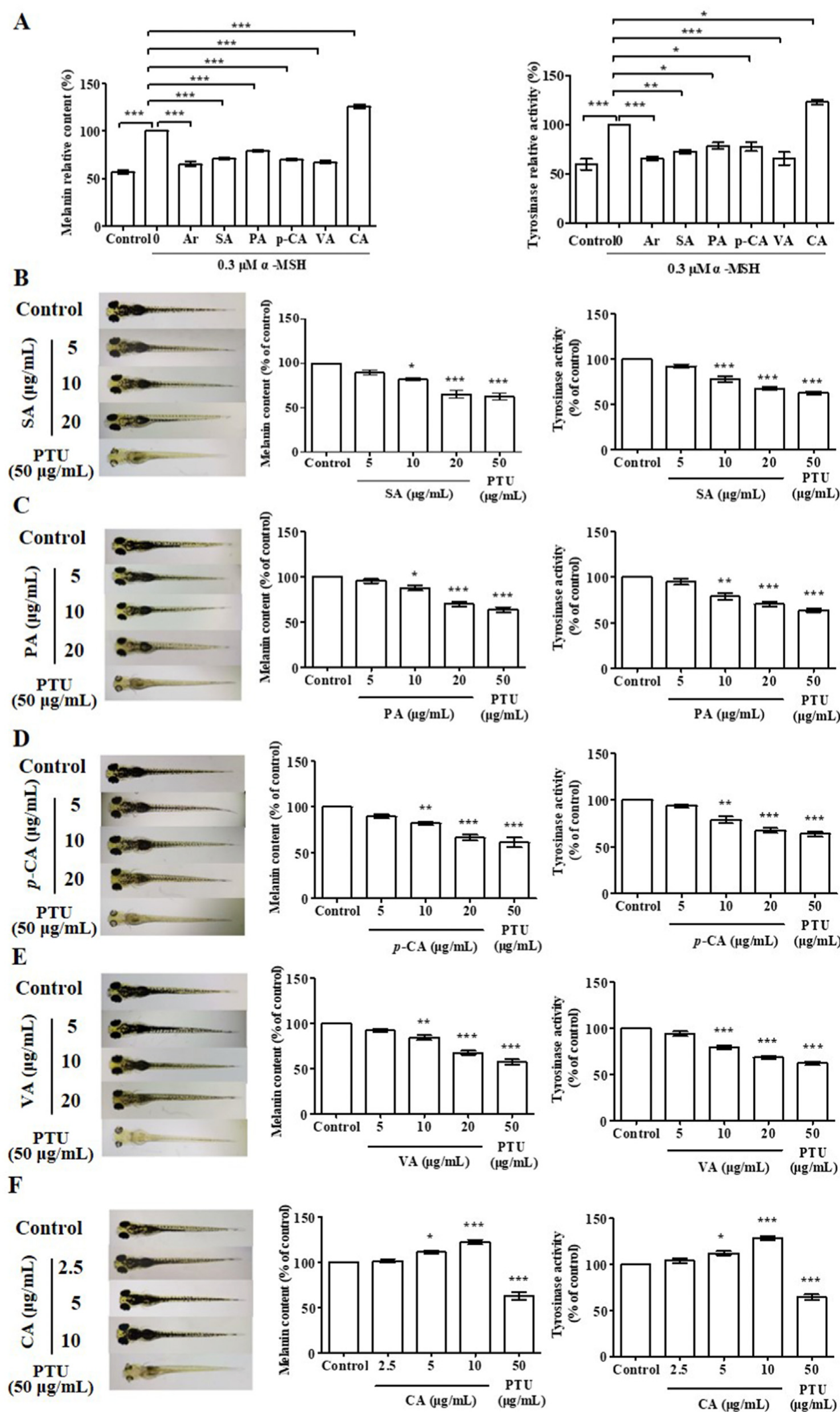

Fig. 1. Effects of *P. ginseng* root phenolic acids on melanin content and tyrosinase activity in α-MSH-stimulated B16F10 cells and zebrafish embryos. (A) Effects of P. ginseng root phenolic acids on melanin content and tyrosinase activity of α-MSH-stimulated B16F10 cells. (B–F) Effect of SA, PA, *p*-CA, VA and CA on the melanin content and tyrosinase activity of zebrafish embryos. Data are all expressed as a numerical value ± standard deviation (n=3 or 50). *p<0.05, **p<0.01, ***p<0.001 for comparisons between two groups.

acids within *P. ginseng* roots can inhibit and promote melanin production.

3.2. SA, PA, p-CA and VA each reduced melanin content and TYR activity in zebrafish embryos, while CA had the opposite effect

Zebrafish is a widely utilized vertebrate model due to its human-like organ system and genome sequence. This investigation utilized zebrafish as in vivo animal model to elucidate depigmentation [36,42,43]. When zebrafish embryos were incubated with a series of concentrations of SA, PA, *p*-CA or VA (Fig. 1B–E), melanin content and TYR activity were markedly reduced dose-dependently, with observed inhibitory effects of positive control (PTU). However, CA treatment increased melanin levels and TYR activity (Fig. 1F). Altogether, both the *in vitro* and *in vivo* investigations indicated that phenolic acid monomeric compounds in *P. ginseng* roots could either inhibit or promote melanin synthesis. The literature indicates that ginsenosides can play different bidirectional regulatory roles, where either single ginsenosides exert opposite regulatory effects on different tissues and in different doses or different ginsenosides exerts opposite regulatory effects on the same system [23–25]. The latter scenario is consistent with bidirectional regulatory observed melanogenic effects of different *P. ginseng* root phenolic acid monomers, as reported here for the first time. We had previously founded that *P. ginseng* phenolic acids inhibited melanogenesis [26], and systematic research the mechanism of SA inhibiting melanogenesis [28], while we found different *P. ginseng* phenolic acid monomers can inhibit or promote the generation of melanin in this research. Combination of different *P. ginseng* root phenolic acid compounds can safely inhibit melanin production via bidirectional regulation through different pathways, which can reduce excessive melanin production without entirely blocking melanogenesis, thereby prevent UV exposure-induced skin damage, becoming a safer and more effective whitening agent.

3.3. SA, PA, p-CA and VA reduced the mRNA and protein levels of melanin synthases in α-MSH-stimulated B16F10 cells, while CA increased mRNA and protein expression of TYR and TYRP1 (but not TYRP2)

To examine the impact of SA, PA, *p*-CA, and VA on the expression of the melanogenesis-associated protein, RT-PCR and western blotting analyses were carried out, which revealed that the mRNA and protein levels of TYR, TYRP1, and TYRP2 in α-MSH-stimulated B16F10 cells were substantially elevated and these levels alleviated after SA, PA, *p*-CA and VA treatment compared to the control group (Fig. 2A–D). Furthermore, CA-treated cells exhibited notably increased mRNA and protein levels of TYR and TYRP1 than untreated α-MSH-stimulated cells (Fig. 2E, F, H); however, TYRP2 mRNA and protein levels were not substantially different from untreated, stimulated cells (Fig. 2G and H). Altogether, these results indicate that SA, PA, *p*-CA, and VA reduces the mRNA and protein expression of TYR, TYRP1, and TYRP2, while CA increases the mRNA and protein expression of TYR and TYRP1 while CA treatment does not affect TYRP-2 expression, suggesting that CA regulate melanin production through different regulatory pathways. Much research found natural products of melanogenesis inhibitors used as skin whitening agents through downregulating MITF expression, and regulating enzymes that are essential for melanin production, including TYR, TYRP-1, and TYRP-2, while CA treatment have no effect on TYRP-2 expression. TYRP1 and TYRP2 catalyze distinct post-TYR reactions in melanin biosynthesis, which suggests that CA regulate melanin production through different regulatory pathways from SA, PA, *p*-CA ,VA and general whitening agents [44,45].

3.4. SA, PA, p-CA and VA each reduced MITF mRNA-level and protein-level expression, but not MITF phosphorylation in α-MSH-stimulated B16F10 cells, while CA treatment increased MITF mRNA-level expression and phosphorylation and reduced MITF protein-level expression

MITF is reported to transcriptionally mediate the substantial impact of α-MSH by regulating enzymes crucial for melanin synthesis, including TYR, TYRP-1, and TYRP-2 [46]. MITF protein posttranslational modifications, such as phosphorylation, influence MITF transcriptional activity. p-MITF can enter the nucleus to promote the TYR, therefore, melanin synthesis is influenced by both MITF mRNA expression and MITF-associated post-transcriptional phosphorylation [47]. As shown in Fig. 2I and J depicts, MITF mRNA, protein and phosphorylation levels in α-MSH-stimulated B16F10 cells were markedly increased than control untreated, stimulated cells. SA, PA, *p*-CA and VA treatments alleviated MITF mRNA and protein levels but did not reduce the p-MITF levels. Moreover, CA-treated α-MSH-stimulated cells exhibited increased MITF mRNA (Fig. 2K) and p-MITF levels and reduced protein levels in a dose-dependent manner than untreated, stimulated cells (Fig. 2L). Therefore, the abovementioned results indicate that SA, PA, *p*-CA and VA inhibited mRNA and protein expression of MITF by decreasing transcription-level expression of melanin synthase to ultimately inhibit melanin synthesis. However, CA treatment did not increase protein levels of MITF but markedly reduced MITF protein even though it promoted MITF mRNA levels and phosphorylation, suggesting that it may promote TYR expression by promoting MITF phosphorylation. Many melanogenesis inhibitors regulated MITF transcription activity and protein expression via CREB phosphorylation, decreasing the expression of TYR, TYRP-1, and TYRP-2, thereby influencing melanin synthesis, their have no reports on affected MITF protein posttranslational modifications. SA, PA, *p*-CA and VA inhibited melanin production through this classical signaling pathways, while CA promotes melanin synthesis through promoting MITF phosphorylation, this may be the reason why *P. ginseng* phenolic acids can inhibit excessive melanin synthesis without limiting it, maintaining its content at a healthy level.

3.5. SA, PA, p-CA and VA inhibited CREB phosphorylation to suppress MITF transcription via the MC1R/cAMP/PKA signaling pathways, while CA promoted MITF transcription of through this pathway

α-MSH can stimulate melanocytes and bind to its MC1R receptors, thereby stimulating AC, promoting intracellular cAMP accumulation, and PKA activation [31,48]. Activated PKA phosphorylates CREB to stimulate MITF transcription and initiate the expression of three major pigmentation enzymes, (i.e., TYR, TYRP1, TYRP2) that synthesis melanin [31,49]. As compared with results obtained for the control group, contents of intracellular cAMP, AC activity, and PKA in α-MSH-stimulated cells were markedly increased, while SA, PA, *p*-CA and VA treatment had alleviating effects (Fig. 3A–C). As shown in Fig. 3D, MC1R and p-CREB in α-MSH-stimulated cells were notably elevated, which reduced after SA, PA, *p*-CA or VA, MC1R expression levels were reduced. However, CA treatment promoted AC activity, cAMP accumulation, PKA activation, and increased p-CREB levels but did not affect MC1R expression (Fig. 3E–H). The abovementioned results indicate that SA, PA, *p*-CA and VA each inhibited cellular CREB phosphorylation, inhibiting MITF transcription via the MC1R/cAMP/PKA signaling pathway to reduce melanin synthesis. By contrast, CA treatment of α-MSH-stimulated cells promoted MITF transcription through the same pathway. MC1R/cAMP/PKA, a classical signaling pathway that

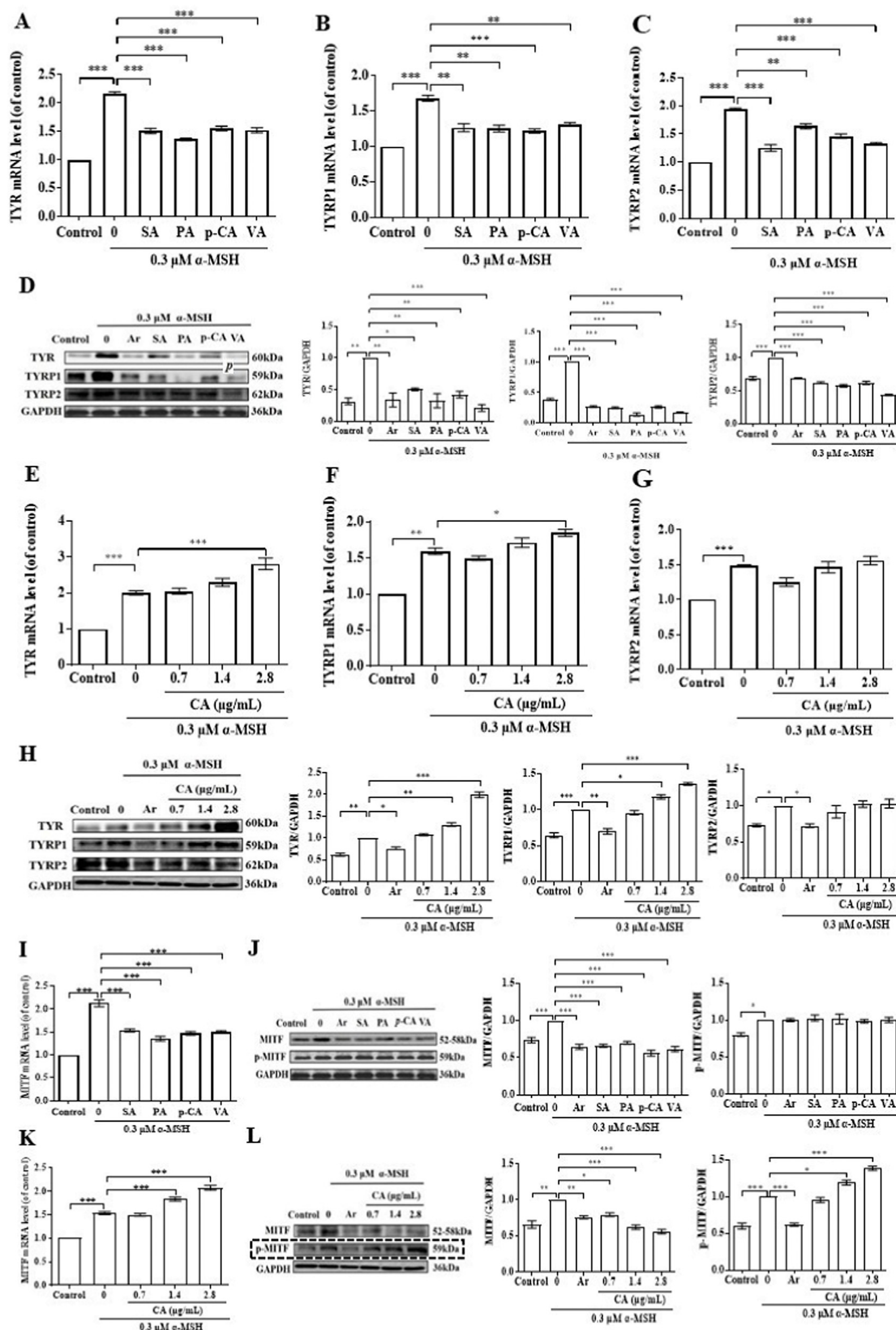

Fig. 2. Effects of *P. ginseng* root phenolic acids on mRNA, protein and phosphorylation levels of melanin synthases in α-MSH-stimulated B16F10 cells. (A-D) Effects of SA, PA, *p*-CA and VA on TYR, TYRP1 and TYRP2 mRNA level and levels of melanin synthase proteins. (E-H) Effect of CA on TYR, TYRP1 and TYRP2 mRNA level and levels of melanin synthase proteins. (I, J) Effects of SA, PA, *p*-CA and VA on MITF mRNA level and MITF protein and phosphorylation levels. (K, L)Effect of CA on MITF mRNA level and MITF protein and phosphorylation levels. Data are all expressed as a numerical value ± standard deviation (n=3). **p*<0.05, ***p*<0.01, ****p*<0.001 for comparisons between two groups.

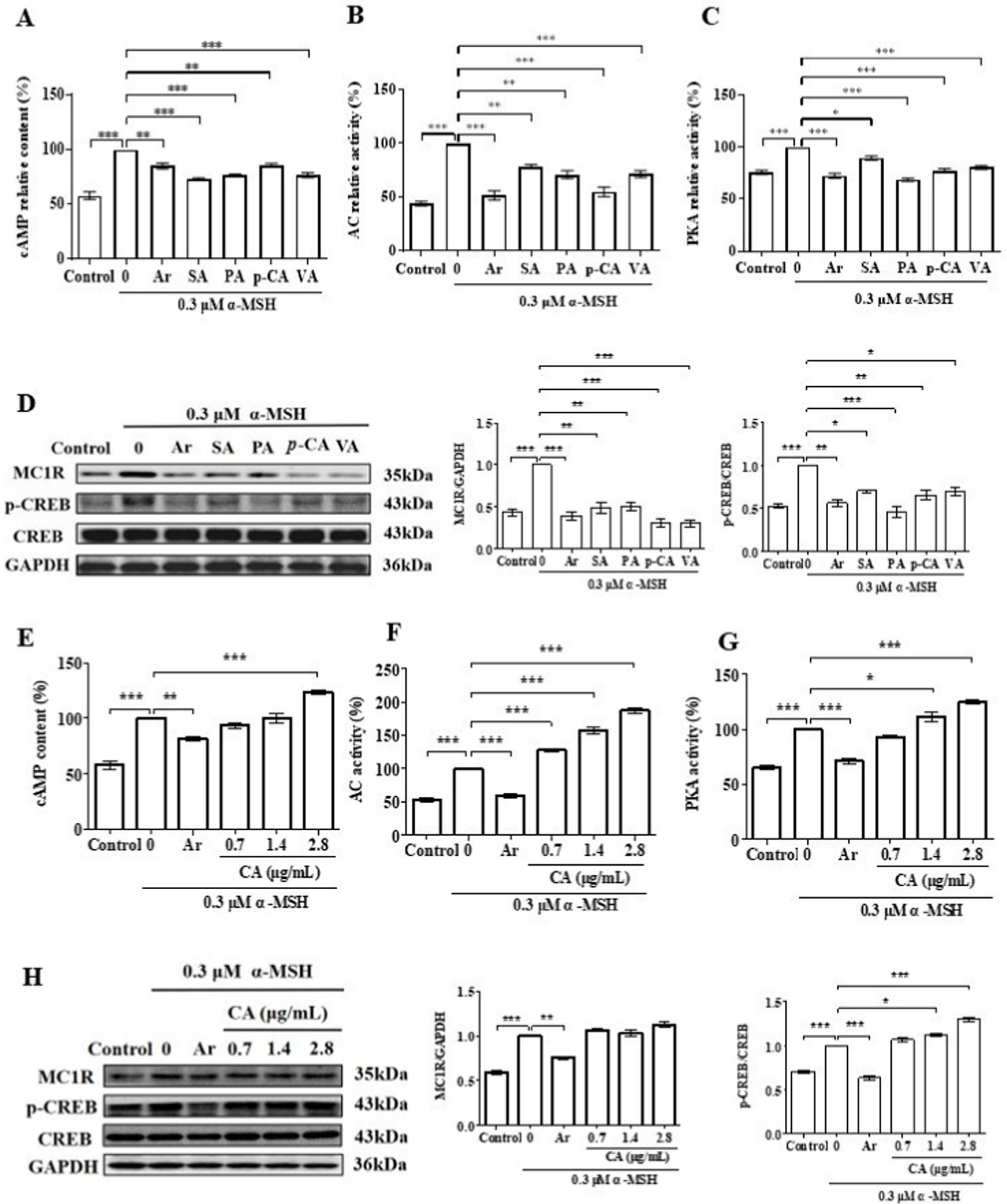

Fig. 3. Effects of *P. ginseng* root phenolic acids on contents, activities and levels of MC1R/cAMP/PKA signaling pathway signal molecules and proteins in α-MSH-stimulated B16F10 cells. (A-C) Effects of SA, PA, *p*-CA and VA on cAMP content, AC and PKA activity. (D) Effect of SA, PA, *p*-CA and VA on protein levels of MC1R and p-CREB/CREB proteins. (E-G) Effect of CA on cAMP content, AC and PKA activity. (H) Effect of CA on protein levels of MC1R and p-CREB/CREB proteins. Data are all expressed as a numerical value ± standard deviation (n=3). **p*<0.05, ***p*<0.01, ****p*<0.001 a for comparisons between two groups.

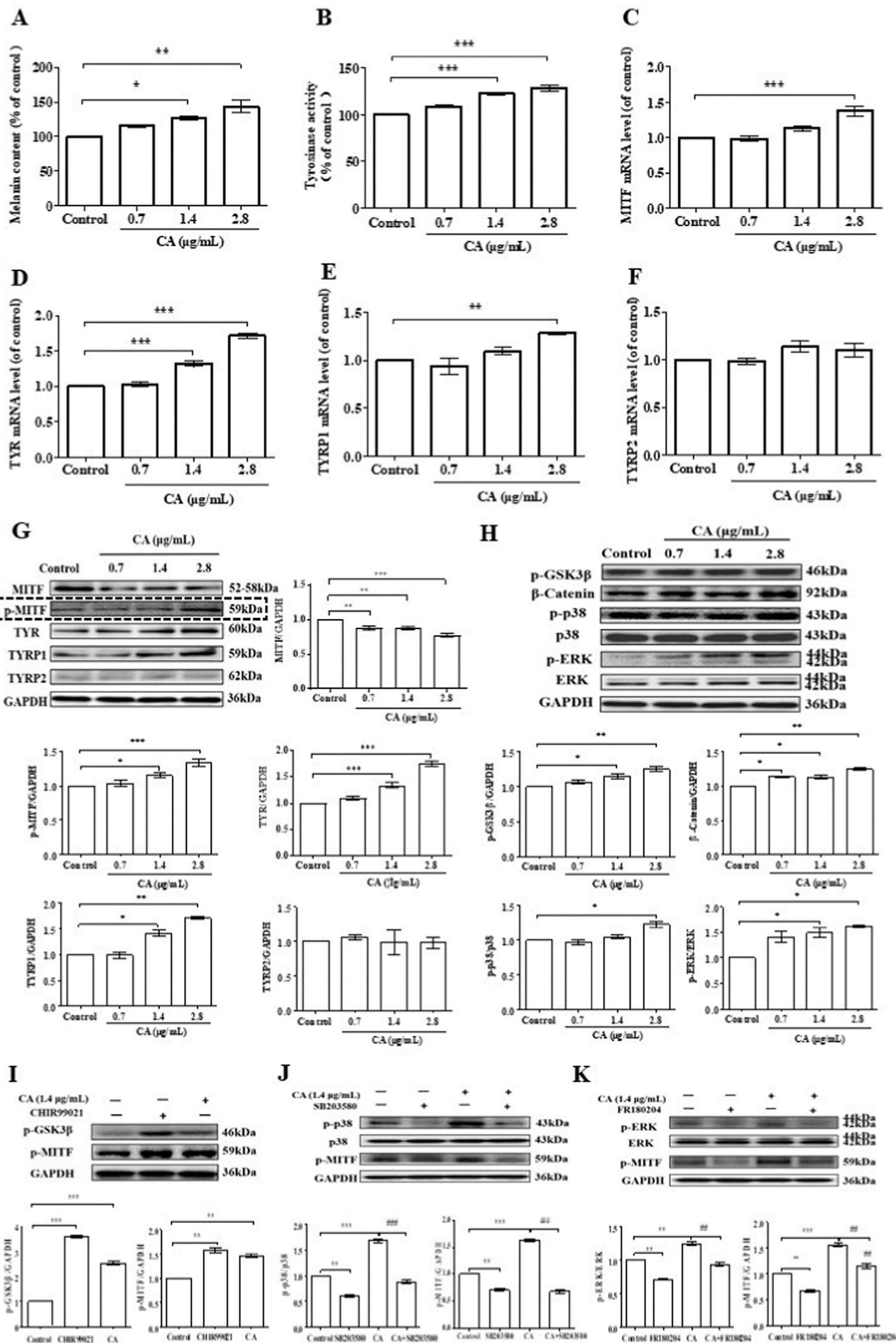

Fig. 4. Effects of CA on melanin synthases and levels of Wnt and MAPK pathways proteins in B16F10 cells. (A, B) Effect of CA on melanin content and tyrosinase activity. (C–F) Effect of CA on MITF, TYR, TYRP1 and TYRP2 mRNA level. (G-K) Effect of CA on melanin synthase protein-level expression and phosphorylation. Data are all expressed as a numerical value ± standard deviation (n=3). *p<0.05, **p<0.01, ***p<0.001 for comparisons between two groups.

regulates melanin synthesis, is suppressed by certain plant-derived compounds, such as kazinol U and calycosin, resulting in inhibition of melanin production [3,50]. In this investigation, SA, PA, *p*-CA and VA reduce MITF transcription that inhibited melanin synthesis by suppressing cAMP/PKA signaling pathway activation of MC1R. These results suggest that the four abovementioned *P. ginseng* root phenolic acid compounds could inhibit melanin production through classical signaling pathways. Intriguingly, CA increased melanin production and promoted cAMP/PKA signaling pathway activity, increasing the mRNA levels of MITF, TYR and TYRP1 and reducing expression of MITF without affecting TYRP2 expression, thus suggesting that CA does not promote melanin production by inducing MITF expression. The above results indicate that *P. ginseng* root phenolic acids can bidirectionally regulate MITF transcription via the cAMP/PKA signaling pathway.

3.6. CA promoted the melanin synthesis by inducing MITF phosphorylation in normal (unstimulated) B16F10 cells

Different CA concentrations were added to normal (unstimulated) B16F10 cells to further elucidate the underlying mechanism for CA's promotion of melanin synthesis. The results revealed that CA treatment markedly increased intracellular melanin content and TYR activity (Fig. 4A and B). Furthermore, it promoted mRNA levels of MITF, TYR, and TYRP1 in B16F10 cells (Fig. 4C–E) but had no notable effect on TYRP2 transcription levels (Fig. 4F). It also stimulated the expression of p-MITF, TYR, and TYRP1 in normal B16F10 cells and inhibited MITF expression, with inhibition becoming gradually more pronounced with increasing CA concentration, while no substantial impact was observed on TYRP2 expression (Fig. 4G). The expressions MITF, TYR, TYRP1, and TYRP2 increased in α-MSH-stimulated B16F10 cells, while CA treated on normal B16F10 cells also promoted melanogenesis. CA regulatory effects observed in normal B16F10 cells were consistent with its effects on α-MSH-stimulated B16F10 cells, indicating that it can regulate melanin synthesis in normal B16F10 cells by modulating the transcription and translation levels of MITF.

3.7. CA increased MITF phosphorylation by modulating Wnt and mitogen-activated protein kinase (MAPK) signal pathway activities

The literature suggests that MAPK signaling pathways (ERK, JNK, and p38) modulate MITF activation, which induces melanin synthesis, the association of GSK-3β/β-catenin signaling with melanogenesis has been reported in many studies [51,52]. The phosphorylation of p38 and c-JNK trigger melanogenesis by stabilizing MITF activation [53,54]. GSK-3β is a negative modulator of the proteins associated with the Wnt/β-catenin signaling pathway and is phosphorylated by multiple kinases [54,55]. Its activation induces β-catenin phosphorylation, then ubiquitination, and finally degradation. Ser9-phosphorylation of GSK-3β inhibits its activity, thereby inhibiting the β-catenin degradation, accumulated cytoplasmic β-catenin translocate into the nucleus, promotes MITF transcription, and eventually promotes the biosynthesis of melanin [55]. To elucidate molecular mechanisms associated with CA effects, the cells were treated with CA with or without inhibitors of Wnt and MAPK signaling pathway proteins. A notable increase in p-GSK3β expression and β-catenin accumulation was observed, which promoted p-p38/p38 and p-ERK/ERK expression (Fig. 4H). Suggesting that CA treatment increased MITF phosphorylation by modulating Wnt and MAPK signal pathways. For further confirmation, inhibitors of these pathways were inoculated with cells, and then MITF phosphorylation levels were assessed. GSK3β inhibitor CHIR99021 promoted the p-GSK3β and p-MITF expression, mirroring CA effects (Fig. 4I). Mechanistically, these results indicated that CA treatment increased GSK3β phosphorylation to promote β-catenin accumulation within the nucleus, ultimately promoting MITF phosphorylation. Additionally, it promoted the expression of p-p38 and p-MITF, which were markedly reduced after adding p38 inhibitors SB203580 (Fig. 4J). Indicating that CA promoted the MITF phosphorylation by increasing p38 phosphorylation. Meanwhile, adding ERK inhibitor FR180204 to cells reduced the CA-induced increase in p-ERK expression, ultimately reducing p-MITF expression (Fig. 4K), suggesting that CA induces MITF phosphorylation by increasing the phosphorylated ERK level. It was revealed that CA treatment promoted the expression of p-MITF, p-GSK3β, β-catenin, p-ERK/ERK, and p-p38/p38, indicating that it may induce MITF post-translational modification to increase the melanin synthesis. Moreover, MITF level is regulated by ERK1/2, GSK3β, and p38 phosphorylation of MITF amino acid residues Ser73, Ser298, and Ser307, respectively, which could enhance its transcriptional activity [15]. GSK3β activation causes β-catenin accumulation and translocation into the nucleus, promoting MITF phosphorylation [56]. Here, CA exerted the same effects as GSK3β inhibitor CHIR99021 by increasing the p-GSK3β expression, reducing GSK3β and p-β-catenin expression, and promoting the β-catenin accumulation and p-MITF expression. Meanwhile, p38 and ERK inhibitors reduced CA-induced increases of p-p38/p38, p-ERK/ERK, and p-MITF expression, suggesting that CA promotes MITF phosphorylation via Wnt/MAPK signaling pathways, also regulated by α-MSH [15,32–34]. Furthermore, it was revealed that when MITF phosphorylation regulated melanin synthase transcription, mRNA and protein levels of TYPR2 did not change. Altogether, CA differed from the other phenolic acid monomers as it promoted MITF transcriptional activity by inducing MITF posttranslational modification.

3.8. Molecular docking and dynamic simulations between SA, PA, p-CA, VA or CA and MC1R

Table 1 and Fig. 5 depict that SA, PA, *p*-CA, VA and CA bind to the catalytic site of MC1R with negative binding energies (−4.5 Kcal/mol, −4.3 Kcal/mol, −4.4 Kcal/mol, −4.7 Kcal/mol, and −4.9 Kcal/mol, respectively) with similar docking scores. The oxygen and carbon atoms of these active compounds formed the different hydrogen bonds with different amino acid residues of MC1R (SA:

Table 1
Ligand Docking Score Sand MC1R Binding Site Residues

Ligand	Docking Score (Kcal/mol)	Hydrogen bond interaction	Hydrophobic interactions	
			π-H	π-π
SA	−4.5	Gln112, Asp115, Leu10		
PA	−4.3	Asp115, Asp119, Leu10		
p-CA	−4.4	Asp119	Lys8	
VA	−4.7	Asp115, Asp119, Leu10	Lys8	
CA	−4.9	Asp115, Asp119	Lys8	Phe278

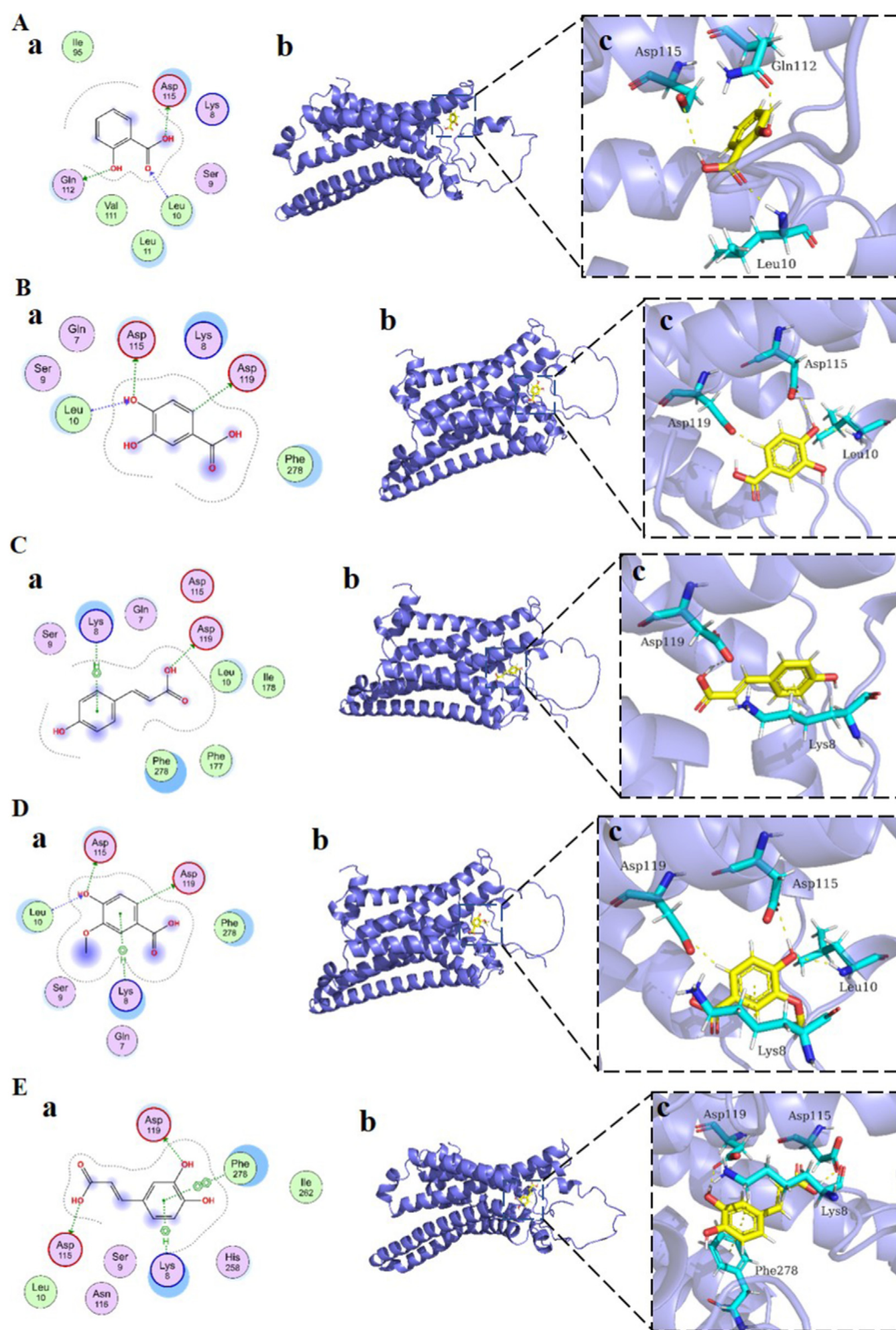

Fig. 5. Binding modes of SA, PA, *p*-CA, VA and CA with MC1R. (A) The binding mode of SA with MC1R; (a) The 2D binding mode of SA with MC1R; (b) The surface binding mode of SA with MC1R; (c) The 3D binding mode of SA with MC1R with SA coloured yellow, surrounding binding pocket residues coloured cyan, the receptor backbone depicted as a light-blue drawing, hydrogen bonds depicted as yellow dashed lines. (B-E) The binding mode of PA, *p*-CA, VA and CA with MC1R with (a), (b) and (c) are the same as above.

Gln112, Asp115, Leu10; PA: Asp115, Asp119, Leu10; p-CA: Asp119; VA: Asp115, Asp119, Leu10; CA: Asp115, Asp119). Furthermore, *p*-CA, VA and CA had in π-H interactions with Lys8 in MC1R, whereas CA had a unique π-π interaction with MC1R Phe278. Altogether, these monomers interact with different MC1R catalytic site residues via different types of chemical bonds that may be related to their different bidirectional regulatory effects. Through molecular docking, it was observed that CA bind MC1R to trigger melanin

synthesis through a mechanism that did not promote MITF expression. Notably, differences in CA versus SA, PA, p-CA, and VA effects might be linked with specific MC1R sites (e.g., π-π, Phe278) bound by CA compared to MC1R sites bound by the other four phenolic acids.

4. Conclusions

Different *P. ginseng* root phenolic acid compounds, when used together can safely inhibit melanin synthesis vis bidirectional regulation of TYR gene transcription through different pathways. SA, PA, *p*-CA and VA, phenolic acid monomers, inhibited TYR transcription via MC1R/cAMP/PKA signaling pathway by reducing MITF expression, which inhibits TYR synthesis and reduces melanin production. However, CA phenolic acid monomer modulated Wnt/MAPK signaling pathway to promote MITF protein level that increased posttranslational MITF phosphorylation to promote TYR gene transcription, increasing melanin production.

Author agreement

The authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

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