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Effect of Standardized *Boesenbergia pandurata* Extract and Its Active Compound Panduratin A on Skin Hydration and Barrier Function in Human Epidermal Keratinocytes

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ABSTRACT: The skin plays a key role in protecting the body from the environment and from water loss. Cornified envelope (CE) and natural moisturizing factor (NMF) are considered as the primary regulators of skin hydration and barrier function. The CE prevents loss of water from the body and is formed by cross-linking of several proteins. Among these proteins, filaggrin is an important protein because NMF is produced by the degradation of filaggrin. Proteases, including matriptase and prostasin, stimulate the generation of filaggrin from profilaggrin and caspase-14 plays a role in the degradation of filaggrin. This study elucidated the effects of an ethanol extract of *Boesenbergia pandurata* (Roxb.) Schltr., known as fingerroot, and its active compound panduratin A on CE formation and filaggrin processing in HaCaT, human epidermal keratinocytes. *B. pandurata* extract (BPE) and panduratin A significantly stimulated not only CE formation but also the expression of CE proteins, such as loricrin, involucrin, and transglutaminase, which were associated with PPARα expression. The mRNA and protein levels of filaggrin and filaggrin-related enzymes, such as matriptase, prostasin, and caspase-14 were also up-regulated by BPE and panduratin A treatment. These results suggest that BPE and panduratin A are potential nutraceuticals which can enhance skin hydration and barrier function based on their CE formation and filaggrin processing.

Keywords: *Boesenbergia pandurate* (Roxb.) Schltr., fingerroot, panduratin A, skin hydration, natural moisturizing factor

INTRODUCTION

Stratum corneum (SC), the outermost layer of the epidermis, protects the organism against external environmental damage and water loss (1). The SC is composed of corneocytes which are terminally differentiated to keratinocytes; thereby, keratinocyte differentiation is required for skin hydration and barrier function (2). Corneocytes are surrounded by an insoluble protein structure, also known as cornified envelope (CE), and a high concentration of natural moisturizing factors (NMF) exists within the corneocytes. These two factors play a pivotal role in skin hydration and barrier function (3,4). Therefore, dysfunction of CE and NMF leads to defective skin hydration and barrier function and also affects skin diseases, such as psoriasis, atopic dermatitis, and ichthyosis vulgaris (5).

The CE is a structure that prevents loss of water from the body, and it is formed by cross-linking of several proteins. A calcium dependent enzyme, called transglutaminase, contributes to this process. Specifically, transglutaminase catalyzes the formation of the isopeptide bond between proteins (6). Main substrates of transglutaminase are CE proteins, such as involucrin, loricrin, and filaggrin. The expression of transglutaminase, involucrin, loricrin, and filaggrin is increased with epidermal barrier formation (6). Several studies showed that peroxisome proliferator-activated receptors (PPARs), a family of nuclear hormone receptors, are closely associated with epidermal homeostasis. The PPARs have three types of subunits, α, γ, and δ. Among these subunits, PPARα has been reported to improve skin health through human epidermal differentiation and hydration (7-9).

Filaggrin is a key marker of epidermal barrier function and skin hydration because filaggrin processing generates NMF (10,11). In the human epidermis, filaggrin exists as the precursor protein, profilaggrin, consisting of filaggrin repeats (11). Profilaggrin is cleaved by proteases, such as matriptase and prostasin, which convert

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profilaggrin into active filaggrin monomers participating in CE formation (12,13). In addition, filaggrin is degraded by caspase-14, generating free amino acids and derivatives like urocanic acid and pyrrolidone carboxylic acid. These amino acid components are the main constituents of NMF, which plays a crucial role in water holding capacity of SC (10,14).

Boesenbergia pandurata (Roxb.) Schltr. is a tropical plant that has been used as a spice and in traditional medicine. *B. pandurata* contains various bioactive compounds, such as pinostrobin, cardamonin, boesenbergin, 5,7-dihydoxyflavone, 1,8-cineole, and panduratin A (15). The active compound panduratin A has biological activities, such as anti-inflammation, anti-oxidation, anti-bacteria, and anti-obesity (15). In addition, the anti-photoaging effect of *B. pandurata* extract (BPE) and panduratin A has been reported in dermal fibroblast and hairless mice (16,17); however, their effects on skin hydration in human epidermal keratinocytes remain to be elucidated. In the present study, we report whether BPE and panduratin A improve skin hydration and barrier function by assessing CE formation and filaggrin processing in HaCaT, human epidermal keratinocytes.

MATERIALS AND METHODS

Preparation of standardized BPE and panduratin A

Rhizomes of *B. pandurata* were collected in Jakarta, Indonesia. A specimen voucher has been deposited in the Department of Biotechnology at Yonsei University (Seoul, Korea). Dried fingerroot rhizomes were ground and extracted with 95% ethanol for 3 days at room temperature. The BPE was obtained by filtration followed by solvent evaporation (extraction yield was 12.0%). The standardized BPE contained 8% (w/w) panduratin A as the bioactive compound.

Isolation of panduratin A

Panduratin A (Fig. 1) was isolated from the 95% ethanol extract of *B. pandurata*, according to the method of Shim et al. (16). The purity of panduratin A was determined to be ≥98% by HPLC analysis (Japan Analytical Industry Co., Ltd., Tokyo, Japan). The isolated panduratin A was dissolved in dimethyl sulfoxide (DMSO) and further diluted in differentiation medium for all of the *in vitro* assays.

Cell culture

The HaCaT, immortalized human keratinocytes, were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories, Inc., Logan, UT, USA) supplemented with penicillin (120 units/mL), streptomycin (75 μL), and 10% fetal bovine serum (FBS; Hyclone Laboratories, Inc.) in an atmosphere of 5% CO_2 at 37°C. The HaCaT cells were maintained until 80% confluence and then the cells were treated with various concentrations of BPE (5, 10, and 20 μg/mL) and panduratin A (0.1, 0.5, and 1 μM) in serum-free medium and incubated for 24 h.

Cell viability

Cell viability was determined with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, MO, USA) colorimetric assay. The HaCaT cells were cultured in 24-well plates (1×10^5 cells/well) for 24 h. The cells were treated with various concentrations of BPE (5, 10, 20, 30, and 40 μg/mL) and panduratin A (1, 3, 5, 10, and 20 μM), and incubated for 24 h in serum-free medium. The cell medium was replaced with 300 μL of MTT reagent (0.5 mg/mL) and incubated for an additional 4 h. After washing the cells, the insoluble formazan products were dissolved in 200 μL dimethyl sulfoxide (DMSO). Absorbance at 540 nm was determined by spectrophotometry using a Versa Max tunable microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA). The BPE concentrations under 20 μg/mL and panduratin A concentrations under 1 μM did not affect cell viability as compared to the control (Fig. 2). Thus, further studies were carried out at BPE concentrations under 20 μg/mL and panduratin A under 1 μM.

Cornified envelope (CE) formation assay

The ability of BPE and panduratin A to induce CE formation was examined in cultured keratinocytes according to the protocol described previously (18,19). Cells were detached with trypsin, and the cell suspension was dispensed into eppendorf tubes and centrifuged. A solution of 2% sodium dodecyl sulphate (SDS), 20 mM dithiothreitol (DTT) and 0.1 M Tris buffer was added to the cell pellets and boiled for 5 min. The amounts of in-

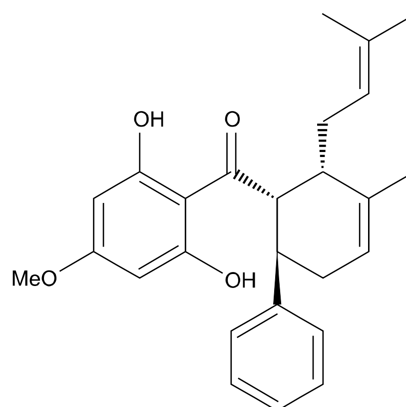

Fig. 1. Chemical structure of panduratin A.

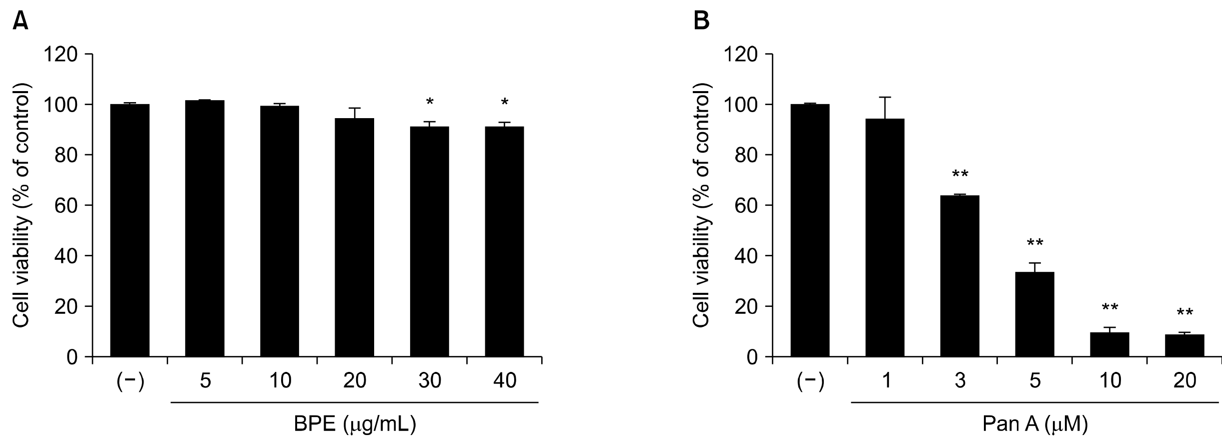

Fig. 2. Cytotoxicities of BPE and panduratin A in HaCaT cells. (A) Cytotoxicity of BPE. (B) Cytotoxicity of panduratin A. HaCaT cells were pretreated with various concentrations of BPE and panduratin A for 24 h. Cell viability was determined by the MTT assay. Results are expressed as mean±SD (% of control) of three independent experiments; $^{*}P<0.05$, $^{**}P<0.01$.

soluble cross-linked envelopes were determined by measuring the absorbance at 340 nm using a Versa Max tunable microplate reader (Molecular Devices Inc.).

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from the cell pellet with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and quantified by spectrophotometry at 260 nm. The cDNA was synthesized in a 20 μL reaction containing 1 μg total RNA, oligo (dT), and Reverse Transcription Premix (Elpis Biotech, Daejeon, Korea). The PCR amplification of the cDNA products (5 μL) was performed with a PCR premix (Elpis Biotech) and the following primer pairs (Bioneer, Daejeon, Korea): filaggrin forward 5'-AGT GCA CTC AGG GGG CTC ACA-3', filaggrin reverse 5'-CCG GCT TGG CCG TAA TGT GT-3' (516 bp); involucrin forward 5'-GGG GCA GCT GAA GCA CCT GG-3' and involucrin reverse 5'-GAG ACG GGC CAC CTA GCG GA -3' (274 bp); loricrin forward 5'-GGG TAC CAC GGA GGC GAA GGA-3', loricrin reverse 5'-ACT GAG GCA CTG GGG TTG GGA-3' (203 bp); prostasin forward 5'-GCT CGC TTC GGA TAC TCC AG-3', prostasin reverse 5'-CTC AGA CAC GAG AGA GCC AC-3' (433 bp); matriptase forward 5'-CTC AAT CTC CAG GGC TCC AAA-3', matriptase reverse 5'-ATA CAC ACA CTG AAG TCA CCT GGG-3' (379 bp); glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward 5'-TGA CCT TGG CCA GGG GTG CT-3', GAPDH reverse 5'-CCA CCC GAG CCA CAT CGC TC-3' (517 bp). Before PCR amplification, primers were denatured at 94°C for 5 min. Amplification consisted of more than 22 cycles: denaturation at 94°C for 30 s, annealing at 56°C for 1 min, and extension at 72°C for 1 min, followed by a final 5 min extension at 72°C. The PCR was performed in a Gene Amp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). The PCR products were separated by 1.5% agarose gel electrophoresis and stained with loading star (Dyne Bioinc, Seoul, Korea). GAPDH was used as an internal control.

Western blot analysis

The HaCaT cells were lysed in RIPA lysis buffer (Elpis Biotech) with a protease inhibitor cocktail (Sigma-Aldrich). Lysate protein concentrations were determined by the Bradford assay. Western blot analysis was then performed as previously described (20). The blots were incubated at 4°C with antibodies against PPARα, loricrin, involucrin, transglutaminase, filaggrin, matriptase, prostasin, caspase-14 (1:500 to 1:1,000 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and α-tubulin (1:1,200 dilution; Cell Signaling, Beverly, MA, USA). Bound antibodies were detected with a horseradish peroxidase-conjugated secondary antibody (1:5,000 dilution; Bethyl Laboratories, Inc., Montgomery, TX, USA). Signals were detected with the enhanced chemiluminescence (ECL) detection system (Amersham BioSciences UK Ltd., Amersham, Buckinghamshire, UK) and visualized with G:BOX EF imaging system (SynGene, Cambridge, UK) and the GeneSnap (SynGene).

Statistical analysis

All experiments were repeated at least three times, and each experiment was performed in triplicate. Results are presented as mean±standard deviation (SD). Statistical analyses were performed using SPSS 21.0 (SPSS Inc., Chicago, IL, USA). Group differences were assessed by one-way analysis of variance (ANOVA) followed by Scheffe's multiple comparisons test. $^{*}P<0.05$ and $^{**}P<0.01$ were considered statistically significant.

RESULTS

Effect of BPE and panduratin A on CE formation

The CE is well recognized as a marker of skin hydration and barrier function (19). In this research, the effect of

BPE and panduratin A on CE formation was tested using HaCaT keratinocytes. The CE formation was markedly increased to 20.1% and 35.0% by treatment with 20 μg/mL of BPE and 1 μM of panduratin A, respectively, as compared to the non-treated control (Fig. 3).

Effect of BPE and panduratin A on keratinocyte differentiation markers

The CE is formed during the keratinocyte differentiation process (6). Thus, the mRNA and protein levels of keratinocyte differentiation markers, such as loricrin, involucrin, and transglutaminase were investigated to clarify the ability of BPE and panduratin A to form CE. The mRNA and protein levels of loricrin, involucrin, and transglutaminase were dose-dependently elevated by BPE and panduratin A treatment (Fig. 4 and 5). In addition, BPE and panduratin A increased the protein expression of PPARα regulating epidermal homeostasis. The results indicate that BPE and panduratin A may accelerate keratinocyte differentiation via PPARα.

Effects of BPE and panduratin A on filaggrin formation and degradation

Filaggrin, acting as a key factor for skin hydration, is

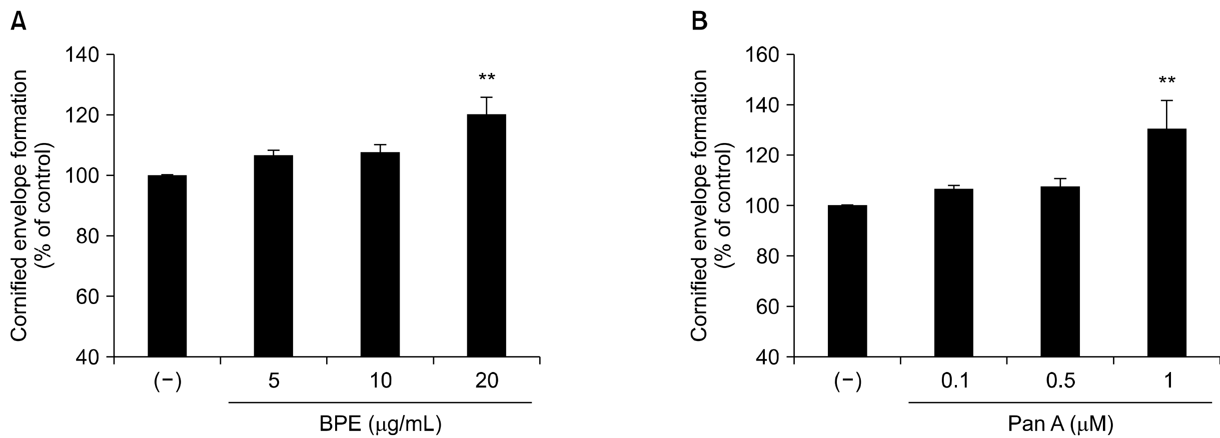

Fig. 3. The effect of BPE and panduratin A on CE formation. HaCaT cells were pretreated with various concentrations of BPE and panduratin A for 96 h. The cells were harvested, and CE was extracted by boiling and quantified by a spectrophotometric method. (A) The effect of BPE on CE formation. (B) The effect of panduratin A on CE formation. Results are expressed as mean±SD (% of control) of three independent experiments; $^{**}P<0.01$.

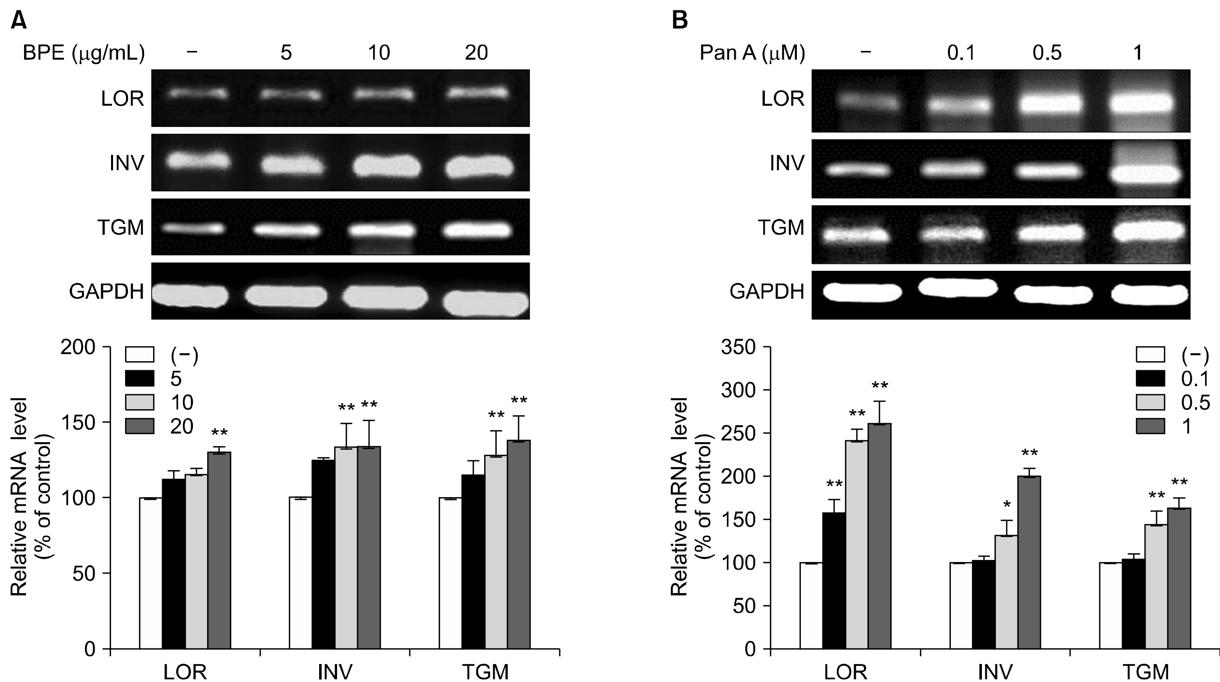

Fig. 4. The effect of BPE and panduratin A on the mRNA expression of loricrin, involucrin, and transglutaminase. The mRNA expression of loricrin, involucrin, and transglutaminase was determined by RT-PCR and quantified by densitometric anlaysis. (A) The mRNA expression of loricrin, involucrin, and transglutaminase in BPE treated HaCaT cells. (B) The mRNA expression of loricrin, involucrin, and transglutaminase in panduratin A treated HaCaT cells. Results are expressed as mean±SD (% of control) of three independent experiments; $^{*}P<0.05$, $^{**}P<0.01$. LOR, loricrin; INV, involucrin; TGM, transglutaminase.

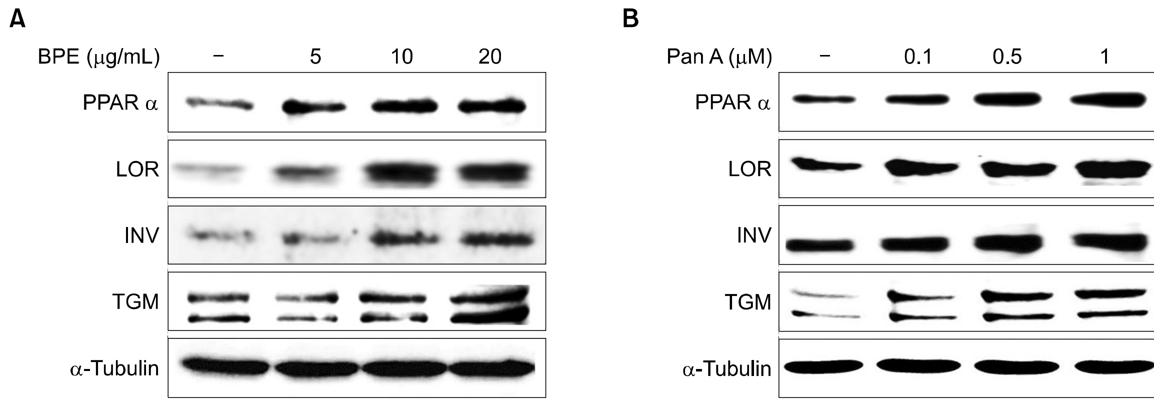

Fig. 5. The effect of BPE and panduratin A on the protein expression of PPARα, loricrin, involucrin, and transglutaminase. The protein expression of PPARα, loricrin, involucrin, and transglutaminase was determined by western blotting analysis. (A) The protein expression of PPARα, loricrin, involucrin, and transglutaminase in BPE treated HaCaT cells. (B) The protein expression of PPARα, loricrin, involucrin, and transglutaminase in panduratin A treated HaCaT cells. LOR, loricrin; INV, involucrin; TGM, transglutaminase.

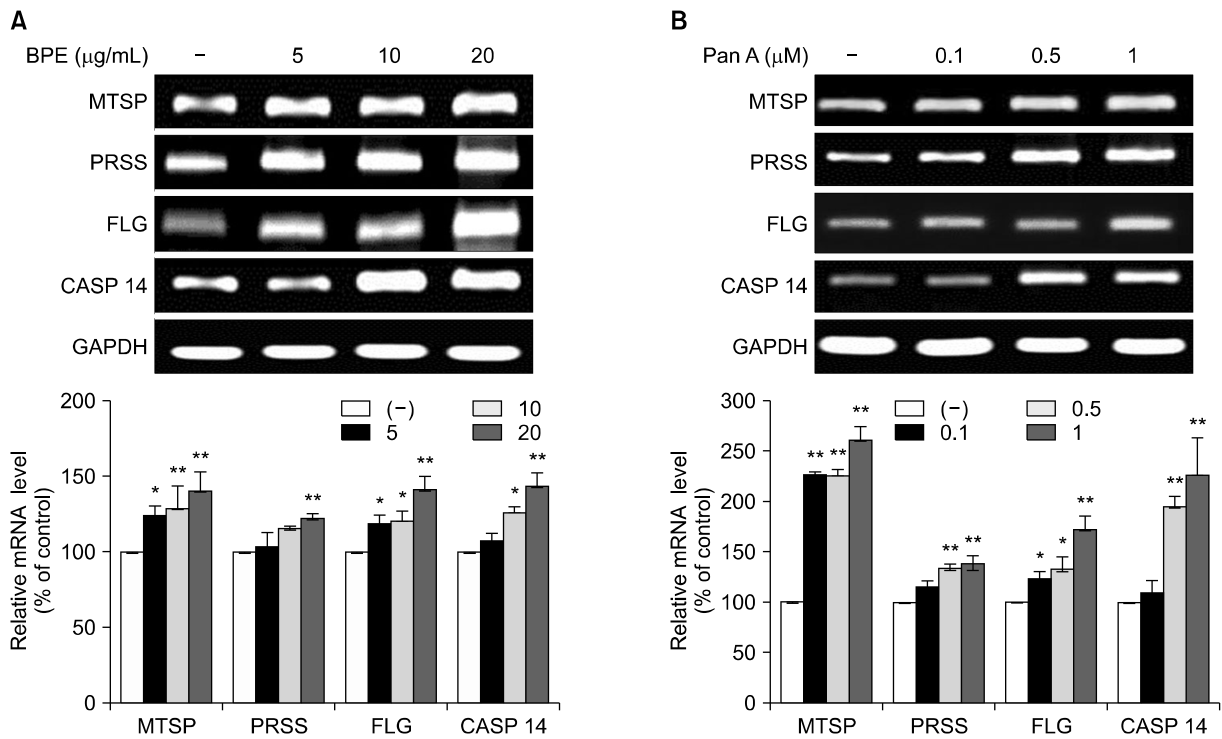

Fig. 6. The effect of panduratin A on the mRNA expression of matriptase, prostasin, filaggrin, and caspase-14. The mRNA expression of matriptase, prostasin, filaggrin, and caspase-14 was determined by RT-PCR and quantified by densitometric anlaysis. (A) The mRNA expression of matriptase, prostasin, filaggrin, and caspase-14 in BPE treated HaCaT cells. (B) The mRNA expression of matriptase, prostasin, filaggrin, and caspase-14 in panduratin A treated HaCaT cells. Results are expressed as mean±SD (% of control) of three independent experiments; *P<0.05, **P<0.01. MTSP, matriptase; PRSS, prostasin; FLG, filaggrin; CASP 14, caspase-14.

generated by proteolysis of profilaggrin. Enzymes, including matriptase and prostasin, contribute to filaggrin production by profilaggrin degradation (12,13). Then, filaggrin monomers are degraded by caspase-14, resulting in NMF production (13). The mRNA expression of matriptase and prostasin was significantly up-regulated by BPE and panduratin A, leading to the increased mRNA expression of filaggrin (Fig. 6). The mRNA level of filaggrin was significantly increased to 41.3% and 71.2% by treatment with 20 μg/mL of BPE and 1 μM of panduratin A, respectively, as compared to the non-treated control. In addition, BPE and panduratin A treatment significantly increased the mRNA expression of caspase-14, an enzyme that produces NMF from filaggrin (Fig. 6). Correspondingly, the protein expression of matriptase, prostasin, filaggrin, and caspase-14 was also markedly increased (Fig. 7). These results demonstrate that BPE and panduratin A stimulate the formation of both filaggrin and NMF by elevating profilaggrin processing enzymes and caspase-14.

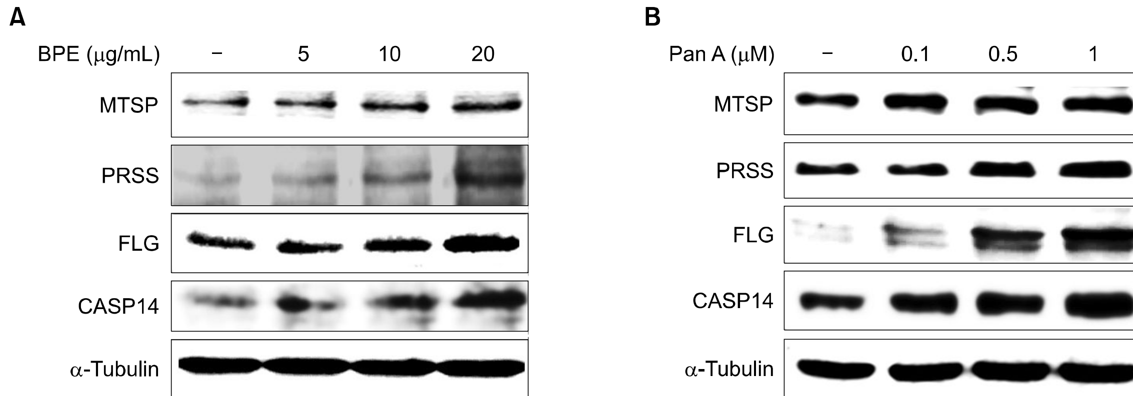

Fig. 7. The effect of panduratin A on the protein expression of matriptase, prostasin, filaggrin, and caspase-14. The protein expression of matriptase, prostasin, filaggrin, and caspase-14 was determined by western blotting analysis. (A) The protein expression of matriptase, prostasin, filaggrin, and caspase-14 in BPE treated HaCaT cells. (B) The protein expression of matriptase, prostasin, filaggrin, and caspase-14 in panduratin A treated HaCaT cells. MTSP, matriptase; PRSS, prostasin; FLG, filaggrin; CASP 14, caspase-14.

DISCUSSION

Epidermal differentiation plays an important role in skin hydration and barrier function (4). During epidermal differentiation, keratinocytes existing in the basal and granular layers of the epidermis differentiate into corneocytes that form SC, the outermost layer of the epidermis. The CE is formed as an exterior part of the corneocytes (2,4). At the highest levels of BPE and panduratin A treatments, CE formation increased by 20.1% and 35.0%, respectively (Fig. 3). The mRNA and protein levels of keratinocyte differentiation markers, such as loricrin, involucrin, and transglutaminase, were also elevated by BPE and panduratin A treatments (Fig. 4 and 5). The BPE (20 μg/mL) significantly increased the expression of involucrin, loricrin, and transglutaminase by 34.2%, 30.1%, and 37.8%, respectively, as compared to the non-treated control. Panduratin A (1 μM) up-regulated the mRNA levels of involucrin, loricrin, and transglutaminase by 160.1%, 99.5%, and 62.3%, respectively, compared to the non-treated control (Fig. 4). Involucrin functions as an early component of CE, attaching to the membrane of keratinocytes during CE formation. Subsequently, crosslinking of loricrin has occurred. Loricrin functions as a reinforcement protein for CE structure, comprising 70~85% of the total protein mass of SC. Crosslinking of proteins is mediated by transglutaminase (6). These results suggest that BPE and panduratin A enhance skin hydration and barrier function through CE formation.

We also investigated the relationship between the receptor system and the epidermal differentiation of BPE and panduratin A. Previous studies demonstrated that the expression of CE proteins was stimulated by the PPARα activators, clofibrate and Wy-14643 (8,9). In addition, PPARα activation enhanced skin hydration with up-regulation of keratinocyte differentiation (21). However, studies of natural PPARα activators on skin hydration and barrier function are very limited. Lim et al. (7) reported that ursolic acid and oleanolic acid enhanced epidermal keratinocyte differentiation via PPARα. In particular, BPE and panduratin A were reported as PPARα/δ dual activators (22,23); however, their effects on PPARα expression in human epidermal keratinocytes is unknown. When HaCaT was treated with BPE and panduratin A, the protein expression of PPARα was significantly increased (Fig. 5). Hence, improvement of skin hydration and barrier function by BPE and panduratin A treatment may be relevant to their PPAR α activation.

The BPE and panduratin A treatments increased the levels of filaggrin through up-regulating the expression of matriptase and prostasin and also the level of caspase-14. Treatments with BPE (20 μg/mL) and panduratin A (1 μM) markedly elevated the mRNA expression of filaggrin by 41.3% and 71.2%, respectively, as compared to the non-treated control (Fig. 6 and 7). Although the processes of filaggrin formation from profilaggrin are not well-established, the role of serine proteases like matriptase and prostasin in filaggrin formation has been well recognized (13). Caspase-14 is involved in the proteolysis of filaggrin, leading to the generation of hygroscopic free amino acids and derivatives, of NMF. The major role of NMF is to maintain skin hydration and water-holding capacity of the corneocytes and to drive acidification of SC (11,12,14). An acidic pH is essential for the antimicrobial effect and enzymatic activity, contributing to ceramide metabolism (12,14).

Taken together, BPE and panduratin A improved skin hydration and barrier function through CE formation and filaggrin processing, suggesting their potential use as natural nutraceuticals to enhance skin health function. Further studies are necessary to elucidate their effects on skin health using animal models and clinical studies.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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