



Panduratin A, an activator of PPAR- α/δ , suppresses the development of oxazolone-induced atopic dermatitis-like symptoms in hairless mice

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ABSTRACT

Aims: Panduratin A isolated from *Boesenbergia pandurata* (Roxb.) has been reported to have antioxidant, anti-inflammatory, and anti-allergic activities. However, the effect of panduratin A on atopic dermatitis (AD) has not been studied. In the present study, we investigated the efficacy of panduratin A, an activator of peroxisome proliferator-activated receptors (PPAR) α/δ , using oxazolone-induced AD-like model in hairless mice.

Main methods: To determine PPAR α/δ activation of panduratin A, HaCaT, Hs68, and COS-7 cells were treated with panduratin A, then PPAR α/δ and PPAR response element (PPRE) activities were assessed with a reporter gene assay. For the in vivo study, oral administration of panduratin A was performed for 4 weeks, with oxazolone treatment every other day. The efficacy of panduratin A on parameters of oxazolone-induced AD was assessed physiologically, morphologically, and immunologically.

Key findings: Panduratin A increased PPAR α/δ and PPRE activation both in vitro and in vivo. Panduratin A attenuated dermatitis-associated barrier damage as demonstrated by transepidermal water loss, erythema, and filaggrin expression. Furthermore, infiltration of inflammatory cells and epidermal thickness in the skin were decreased. Panduratin A decreased serum immunoglobulin (Ig) E and interleukin-4 levels but increased IgG2a and interferon- γ levels. In addition, panduratin A decreased inflammation-associated molecules in the skin. Panduratin A also decreased Th2-associated molecules and increased Th1/regulatory T cell (Treg)-associated molecules in the spleen.

Significance: Panduratin A showed a beneficial effect on AD by modulating Th1/Th2/Treg-associated immune response and is a potential candidate for treating AD.

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Introduction

Atopic dermatitis (AD) is a chronic and relapsing skin disease that is histopathologically characterized by the infiltration of inflammatory cells (Boguniewicz and Leung, 2011). The skin inflammation in AD is partly due to a complicated interaction between altered keratinocytes and activated T helper type 2 cells (Th2), lymphocytes, eosinophils, and mast cells. Furthermore, chronic inflammation causes a decrease in protective epidermal functions, natural moisturizing factors, and antimicrobial protein (Cork et al., 2006). Although the discovery of immunological and genetic disorders in AD has been remarkable in recent years, topical glucocorticoid application is still the most effective therapy and is considered the standard treatment according to several guidelines (Katayama et al., 2011). Prolonged use of topical glucocorticoids could result in side effects such as dry scaly skin, muscle atrophy, and

hypertension (Pels et al., 2008). Therefore, more effective alternative therapies without negative side effects are needed.

Genetic studies have shown that immunologic and skin barrier-related genetic variations may work synergistically to increase susceptibility to AD. AD patients show an increase in transepidermal water loss (TEWL) and a decrease in several other parameters of skin barrier function including dye penetration and stratum corneum hydration (De-Benedetto et al., 2012). Filaggrin is a vital protein that plays an important role in formation of the cornified cell envelope, which is significant for skin barrier homeostasis (Howell et al., 2009). Filaggrin gene deficiencies have recently been determined to be a major risk factor for development of AD (Kubo et al., 2012). Furthermore, the immune modulation function of regulatory T cells (Tregs) is regarded as another important mechanism for immune-associated diseases, such as AD. Tregs, which have an immunosuppressive function, are able to suppress the development of the Th2 response (Szegeedi et al., 2009).

Peroxisome proliferator-activated receptors (PPARs) are a family of nuclear hormone receptors that have three isotypes: PPAR α , δ , and γ . These ligand-activated transcription factors regulate a variety of biological activities including glucose metabolism and homeostasis, insulin

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sensitivity, lipid metabolism, and cell proliferation and differentiation (Varga et al., 2011). Several recent studies have reported that PPARs suppress the expression of inflammatory genes such as cytokines and display positive effects on epidermal function and structure, including upregulation of filaggrin (Schmuth et al., 2008; Demerjian et al., 2009). In addition, PPAR α/δ modulate epidermal homeostasis and nuclear factor-kappa B (NF- κ B) signaling and display broad efficacy in AD (Duan et al., 2009; Hatano et al., 2010). Overall, these studies provide solid evidence for the role of PPAR α/δ in regulating inflammation, suggesting that PPAR α/δ could be potential therapeutic targets for inflammatory diseases and AD.

Panduratin A (Fig. 1) isolated from *Boesenbergia pandurata* (Roxb.) Schltr. and commonly called fingerroot, is a natural chalcone compound that has demonstrated anticancer, antioxidant, anti-inflammatory, antibacterial, and antibiofilm activities (Yun et al., 2003, 2006; Sohn et al., 2005; Rukayadi et al., 2010; Yanti et al., 2009). Panduratin A has been reported to stimulate 5' adenosine monophosphate-activated protein kinase (AMPK) signaling and activate PPAR α/δ in L6 myoblasts (Kim et al., 2011). In addition, panduratin A suppresses degranulation, inflammatory mediator production, and inflammatory cytokine expression in mast cells, demonstrating its potent anti-allergic effects (Choi et al., 2012). However, its effects on AD have not yet been characterized. In the present study, we determined the effects of oral administration of panduratin A, an activator of PPAR α/δ , on AD in an oxazolone-induced hairless mice model.

Materials and methods

Isolation of panduratin A from *B. pandurata*

Dried rhizomes of *B. pandurata* were collected in Jakarta, Indonesia and identified by Dr. Nam-In Baek from the Department of Oriental Medicinal Materials and Processing at Kyunghee University (Yongin, Korea). A voucher specimen was deposited in the Department of Biotechnology, Yonsei University (Seoul, Korea). The dried *B. pandurata* rhizomes (100 g) were ground and extracted with 95% ethanol, and *B. pandurata* extract (BPE) was obtained by filtration followed by solvent evaporation (yield 12.0%). Panduratin A (purity 98.0%) was isolated from 95% ethanol extract of *B. pandurata*, as described previously (Shim et al., 2008).

Cell culture

Human keratinocyte HaCaT cells (passages 8 to 13), monkey kidney COS-7 cells (passages 18 to 21), and human fibroblast Hs68 cells (passages 23 to 26) were obtained from American Type Culture Collection

(Manassas, VA, USA). The cells were grown in Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories, Inc., Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Inc.), streptomycin (75 μ g/ml), and penicillin (120 units/ml) in an atmosphere of 5% CO₂ at 37 °C.

Galactosidase 4 (GAL4)/PPAR chimera transactivation assay

The effect of panduratin A on PPAR α/δ activity was assessed in a GAL4/PPAR α/δ transactivation luciferase assay as described previously (Han et al., 2008). COS-7 monkey kidney cells were cultured in a 24-well plate (4×10^6 cells/well) in DMEM supplemented with 10% FBS for 24 h before transfection with the pFA-hPPAR α/δ expression plasmid and Gal4-driven luciferase reporter pFR-luc (Stratagene). After the 24 h transfection, the medium was changed to serum-free DMEM with panduratin A (0.1 or 1 μ M), PPAR α activator WY14643 (5 μ M; Sigma-Aldrich), or GW501516 (0.1 μ M; Sigma-Aldrich), and the cells were cultured for an additional 24 h. WY14643 (Sigma-Aldrich) or GW501516 (Sigma-Aldrich) was used in DMSO stocks, and the final concentration of vehicle was constant at 0.1%, which had no effect on PPAR α/δ activity. After washing the cells with phosphate-buffered saline (PBS), the cells were lysed with lysis reagent (20 μ l/well; Promega, Madison, WI, USA), and the luciferase assay substrate (Promega) was added. Luciferase activity was measured using a MicroLumatPlus LB 96V luminometer (Berthold, Wildbad, Germany).

HaCaT cells and Hs68 cells were transfected with plasmids containing three PPAR response elements (PPREs), PPRE-tk-luc (plasmid 1015; Addgene, Cambridge, MA, USA). Then, the cells were incubated with panduratin A (1 μ M), WY14643 (5 μ M; Sigma-Aldrich) or GW501516 (0.1 μ M; Sigma-Aldrich) for 24 h. Luciferase activity was measured as described above.

Animals

Specific pathogen-free 5-week-old female SKH-1 hairless mice (Central Lab., Seoul, Korea) were housed in a controlled environment (22 ± 2 °C, $55 \pm 15\%$ relative humidity, 12 h light/dark cycle). After a one-week acclimation, mice were fed a standard diet (Cargill Agri Purina, Seongnam, Korea) and water. Animal experiments were approved by the Institutional Animal Care and Use Committee at Yonsei University in Seoul, Korea.

Induction of AD-like skin lesions and sample treatment

Mice were topically treated with 200 μ l of 0.5% oxazolone (Sigma-Aldrich, St. Louis, MO, USA) to the dorsal skin three times a week for two weeks. Induction of AD was confirmed through macroscopic observation (e.g., eruption and erythema). After AD-like skin lesions were induced for 2 weeks, the 40 mice were divided into six experimental groups; group 1 received vehicle (polysorbate 80) without oxazolone treatment for 4 weeks ($n = 8$); group 2 received vehicle (polysorbate 80) with oxazolone treatment for 4 weeks ($n = 8$); group 3 received low-dose panduratin A (PAN10; 10 mg/kg/day) with oxazolone treatment for 4 weeks ($n = 8$); group 4 received high-dose panduratin A (PAN25; 25 mg/kg/day) with oxazolone treatment for 4 weeks ($n = 8$); and group 5 received dexamethasone (DEX; 2.5 mg/kg/day) with oxazolone treatment for 4 weeks ($n = 4$). All mice except those in group 1 were topically treated with 200 μ l of 0.2% oxazolone to the dorsal skin three times a week for 4 weeks (total of 12 challenges). Mice in groups 3–6 were also fed PAN10, PAN25, or DEX daily for 4 weeks using oral feeding needles in addition to topical oxazolone treatment. At the end of the 4-week oral administration period, TEWL and erythema were measured, and blood was collected from the retro-orbital plexus. After the experiments, 12-week-old mice were sacrificed using CO₂ gas. Spleens and skin lesions were removed and stored at -80 °C until analysis.

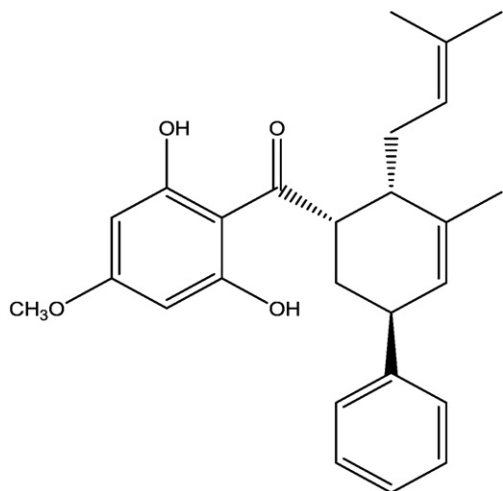


Fig. 1. Chemical structure of panduratin A.

Analysis of TEWL and erythema

To assess skin barrier function, TEWL was measured using a Tewameter TM 300 (Courage & Khazaka, Cologne, Germany), and erythema was measured using a Mexameter MX 18 (Courage & Khazaka) according to the manufacturers' instructions. Measurements were performed in a controlled environment (22 ± 2 °C, $55 \pm 15\%$ relative humidity).

Histological and immunohistochemical analyses

Skin lesions were fixed with 10% neutral formalin, embedded in paraffin, and sectioned at 5 μ m. To detect epidermal thickness and infiltration of inflammatory cells including mast cells, sections were stained with hematoxylin–eosin (H&E) and toluidine blue. Filaggrin expression was detected using polyclonal rabbit anti-filaggrin (Abcam, Edinburgh, UK) as a primary antibody at 1:200 overnight at 4 °C and secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h. All stained sections were observed by an Eclipse TE2000U Inverted Microscope with twin CCD cameras (magnification, $\times 200$; Nikon, Tokyo, Japan).

Quantitative morphology

The number of mast cells in toluidine blue-stained sections and the epidermal thickness in H&E-stained sections were measured from more than 10 fields in each sample using AxioVision software (Carl Zeiss Vision, Jena, Germany). Data are reported as the mean with standard deviation (SD).

Measurement of plasma levels of Igs and cytokines

Blood was collected with a glass capillary tube (Chase Scientific Glass, Rockwood, TN, USA) from the retro-orbital plexus. Serum was obtained by centrifugation at 800 $\times$ g for 10 min and stored at -80 °C until analysis. Total serum IgE levels were determined using a mouse IgE enzyme-linked immunosorbent assay (ELISA) kit (Immunology Consultants Lab., Newberg, OR, USA), and IgG2a levels were measured by the IgG2a ELISA kit (Enzo Life Sciences Inc., Farmingdale, NY, USA). Serum levels of mouse interleukin (IL)-4 (ID Labs Biotechnology Inc., Ontario, NY, USA) and interferon (IFN)- γ (Enzo Life Sciences Inc.) were also measured by ELISA kits according to the manufacturer's instructions.

Table 1
Primers used for RT-PCR.

Gene	Primer	Sequence	Product length (bp)
Mouse IL-1 β	Forward	5'-TCG GCC AAG ACA GGT CGC TC-3'	231
	Reverse	5'-CCT CAG TGC GGG CTA TGA CC-3'	
Mouse IL-5	Forward	5'-GCT GAA GGC CAG CGC TGA AGA-3'	160
	Reverse	5'-ACA GAG CTC GGT GAG CGG ACA-3'	
Mouse IL-10	Forward	5'-CCA GTC GGC CAG AGC CAC AT-3'	208
	Reverse	5'-GGC CAT GCT TCT CTG CCT GGG-3'	
Mouse IL-12a	Forward	5'-TCT CTG GAC CTG CCA GGT GT-3'	542
	Reverse	5'-CCT GTT GAT GGT CAC GAC GCG-3'	
Mouse IL-13	Forward	5'-TGT TTC GCC ACG GCC CCT TC-3'	312
	Reverse	5'-GCT CAA GCT GCT GCC TGC CT-3'	
Mouse IL-31	Forward	5'-TCA GCC TGG ACC GGG AAG CA-3'	325
	Reverse	5'-TCA GCC CTC GAA GGA CAG GC-3'	
Mouse CCL5	Forward	5'-AGG AAC CGC CAA GTG TGTGTG CC-3'	67
	Reverse	5'-AGT GGC ATC CCC AAG CTG GCT A-3'	
Mouse CCL11	Forward	5'-ATG CAC CCT GAA AGC CAT AGT-3'	97
	Reverse	5'-CAG GTG CTT TGT GGC ATC CT-3'	
Mouse CCL17	Forward	5'-CCG AGA GTG CTC CCT GGA TTA-3'	123
	Reverse	5'-AGC TTG CCC TGG ACA GTC AGA-3'	
Mouse CCL22	Forward	5'-GGC ACC TAT CCA GTG CCA CA-3'	134
	Reverse	5'-TGG TGG ACC AGC CTG AAA CTC-3'	
Mouse CCL24	Forward	5'-AAC ATG GCG GGC TCT GCA AC-3'	188
	Reverse	5'-CCT GCC TTG GGA CAG ATG CT-3'	
Mouse CCR3	Forward	5'-AGT GGG CAC CAC CCT GTG AAA-3'	379
	Reverse	5'-CGG GCT CGA AGG GCA AAC ACA-3'	
Mouse CCR5	Forward	5'-GGT GAG ACA TCC GTT CCC CCT-3'	521
	Reverse	5'-ACA GCC ACC GCC CAA GTG AC-3'	
Mouse PAR-2	Forward	5'-CCT GGC CAT GTA CCT GAT CT-3'	217
	Reverse	5'-GAC ACT TCG GCA AAG GAG AG-3'	
Mouse TNF- α	Forward	5'-GCG GAG TCC GGG CAG GTC TA-3'	458
	Reverse	5'-GGG GGC TGG CTC TGT GAG GA-3'	
Mouse TGF- β 1	Forward	5'-TGA CGT CAC TGG AGT TGT ACG G-3'	170
	Reverse	5'-GGT TCA TGT CAT GGA TGG TGC-3'	
Mouse T-bet	Forward	5'-CAG CCT GGG GAC GCC CTA CT-3'	280
	Reverse	5'-CAG GTG GTT GCT GAG CGC GA-3'	
Mouse GATA-3	Forward	5'-CGG GTC GGC CAG GCA AGA TG-3'	386
	Reverse	5'-TAA GGG GCC GGT TCT GCC CA-3'	
Mouse Foxp3	Forward	5'-ATC CAG CCT GCC TCT GAC AAG AAC C-3'	401
	Reverse	5'-GGG TTG TCC AGT GGA CGC ACT TGG AGC-3'	
Mouse NF- κ B1	Forward	5'-AGC CGT GCA GCA GAC CAA GG-3'	849
	Reverse	5'-GCC AAG GCG ATG GGT TCC GT-3'	
Mouse NF- κ B2	Forward	5'-CGG ATC TCG ACC TCC ACC GGA-3'	559
	Reverse	5'-ATG CGC ACG AGG TGG GTC AC-3'	
Mouse β -actin	Forward	5'-TAA CCA ACT GGG ACG ATA TG-3'	203
	Reverse	5'-ATA CAG GGA CAG CAC AGC CT-3'	

Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA isolated from the skin and spleen tissues was extracted with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol and quantified by spectrophotometry at 260 nm. The cDNA was synthesized in a 20 μ l reaction containing 2 μ g total RNA, oligo(dT), and reverse transcriptase premix (Elpis-Biotech, Daejeon, Korea). Reverse transcription (RT) was initiated at 70 °C for 5 min, followed by incubation at 42 °C for 55 min, and termination at 94 °C for 5 min. Amplification of the cDNA products (3 μ l) by polymerase chain reaction (PCR) was performed with PCR premix (Elpis Biotech). Oligonucleotide primers specific for mouse cytokines, chemokines, chemokine receptors, and transcription factors are shown in Table 1. Before PCR amplification, primers were denatured at 94 °C for 5 min. Amplification was performed as 32 cycles of 94 °C for 30 s, annealing for 1 min, and extension at 72 °C for 1 min, followed by a final 5 min at 72 °C. PCR amplification of the housekeeping gene beta-actin was performed to control for sample loading and to allow normalization between samples. PCR was performed in a Gene Amp PCR System 2700 (Applied Biosystems, Foster City, CA, USA). PCR products were separated by 1.5% agarose gel electrophoresis and visualized by ethidium bromide staining and UV illumination. A densitometer was used for RT-PCR on DIG chemiluminescent film (volume of all markers/volume of β -actin).

Statistical analysis

Results are expressed as mean with SD. Groups were compared by one-way analysis of variance followed by the Scheffé test. SPSS computer program (Version 12.0; SPSS Inc., Chicago, IL, USA) was used for data analysis. # P < 0.05, ## P < 0.05, * P < 0.05, and ** P < 0.01 were considered significant.

Results

The effects of panduratin A on PPAR α / δ activation

To evaluate the effects of panduratin A on PPAR α / δ activity, the Gal4/PPAR α / δ transactivation assay was used. COS-7 cells were transfected with pFA-hPPAR α and treated with panduratin A. Panduratin A dose-dependently elevated PPAR α luciferase activity (Fig. 2A). In COS-7 cells transfected with pFA-hPPAR δ , panduratin A also dose-dependently increased PPAR δ luciferase activity (Fig. 2B). To explore the potential use of panduratin A as a PPAR α / δ activator, transient transfections were performed in HaCaT cells and Hs68 cells. Panduratin A stimulated PPRE-dependent luciferase activities in transfected cells (Fig. 2C), and the activity was comparable to that of WY14643 (1 μ M) and GW501516 (0.1 μ M).

The effects of panduratin A on clinical and histological AD symptoms and infiltration of inflammatory cells

Improvement in clinical skin condition after oral administration of panduratin A was confirmed by analysis of H&E-stained and toluidine blue-stained sections. The group treated with oxazolone showed noticeable hyperkeratosis (Fig. 3A), marked epidermal thickening (Fig. 3B), and infiltration of inflammatory cells, particularly mast cells (Fig. 3C). However, mice fed panduratin A for 4 weeks had suppressed epidermal thickening and reduced infiltration of inflammatory cells. These results indicate that oral administration of panduratin A could effectively inhibit the development of dermatitis in hairless mice.

The effects of panduratin A on skin barrier function

To evaluate the effect of panduratin A on skin barrier function, TEWL and erythema were evaluated. Although oxazolone prominently increased TEWL and erythema, mice that received panduratin A by oral

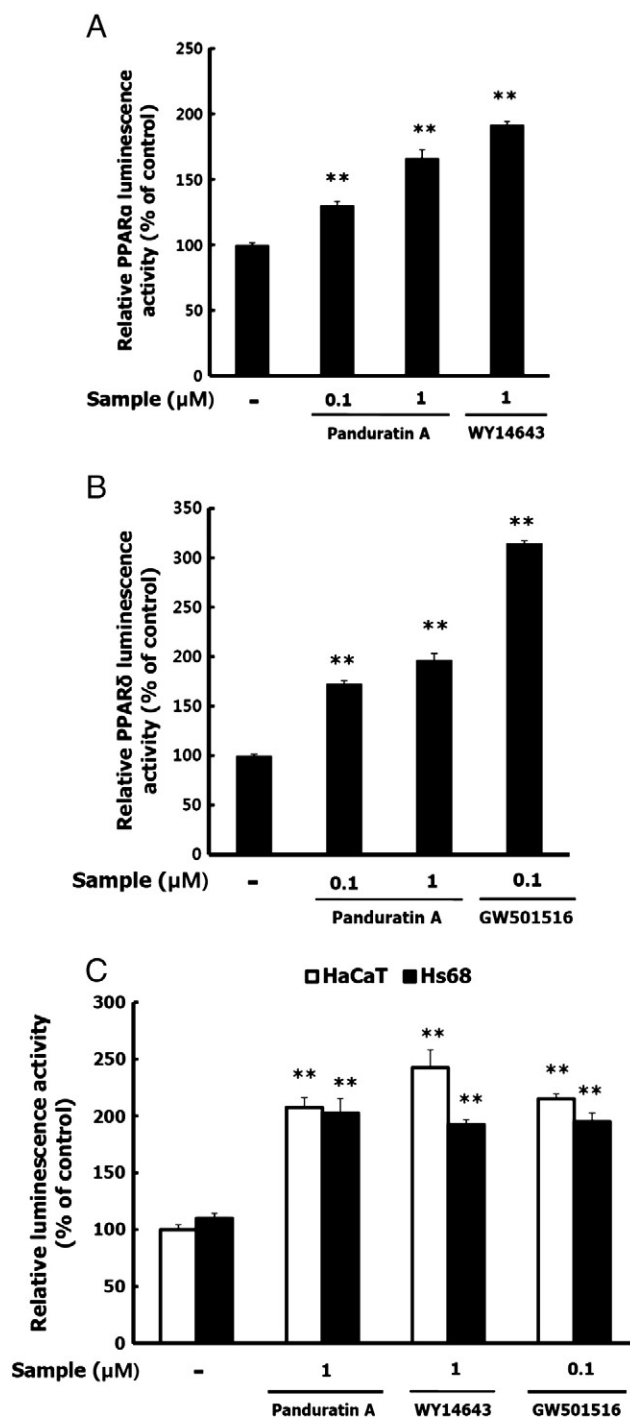


Fig. 2. Effects of panduratin A on PPAR α / δ activation in COS-7 cells (A, B), and PPRE activation in HaCaT and Hs68 cells (C). Results are expressed as mean $\pm$ SD of three independent experiments. # P < 0.05 and ## P < 0.01 compared with vehicle group; * P < 0.05 and ** P < 0.01 compared with oxazolone-treated group.

administration revealed markedly reduced TEWL and erythema in skin lesions (Fig. 4A and B). These barrier protective effects of panduratin A in AD-like skin lesions were further evidenced by the reversal of AD-associated expression changes in the keratinocyte differentiation marker, filaggrin. In the oxazolone treatment group, epidermal filaggrin immunostaining was reduced as compared with vehicle group. However, in panduratin A-fed mice, the immunoreactivity of filaggrin became much more prominent (Fig. 4C). These results indicate

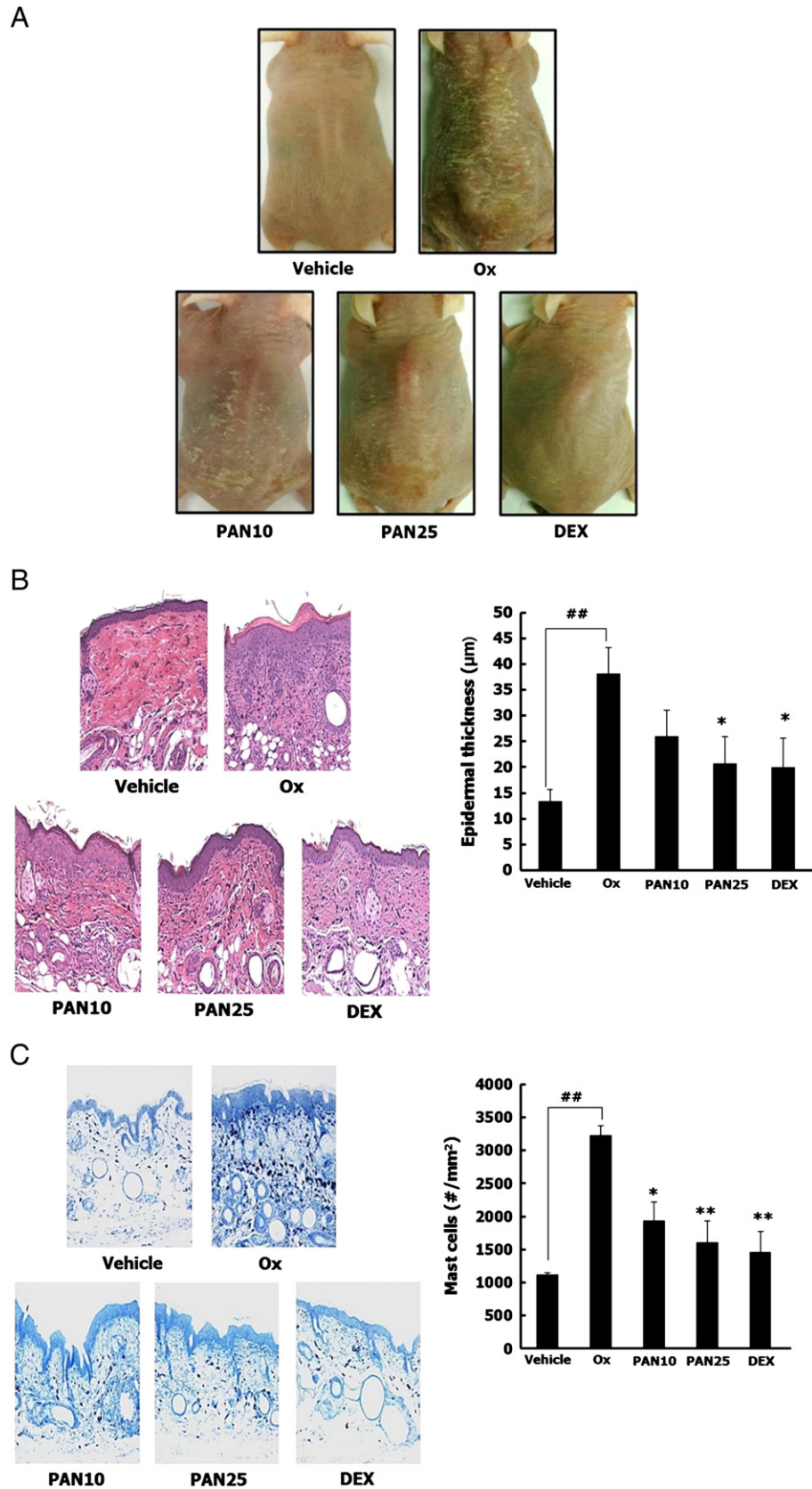


Fig. 3. Effects of panduratin A on gross appearance (A), H&E staining and epidermal thickness (B), and toluidine blue staining and the number of mast cells (C) of oxazolone-treated hairless mice. Mice received vehicle without oxazolone treatment (vehicle group), vehicle with oxazolone treatment (Ox group), 10 mg/kg/day panduratin A with oxazolone treatment (PAN10 group), 25 mg/kg/day panduratin A with oxazolone treatment (PAN25 group), or 2.5 mg/kg/day DEX with oxazolone treatment (DEX group). Results are expressed as mean $\pm$ SD ($n = 5$). $^{##}P < 0.01$ compared with vehicle group; $^*P < 0.05$ and $^{**}P < 0.01$ compared with oxazolone-treated group.

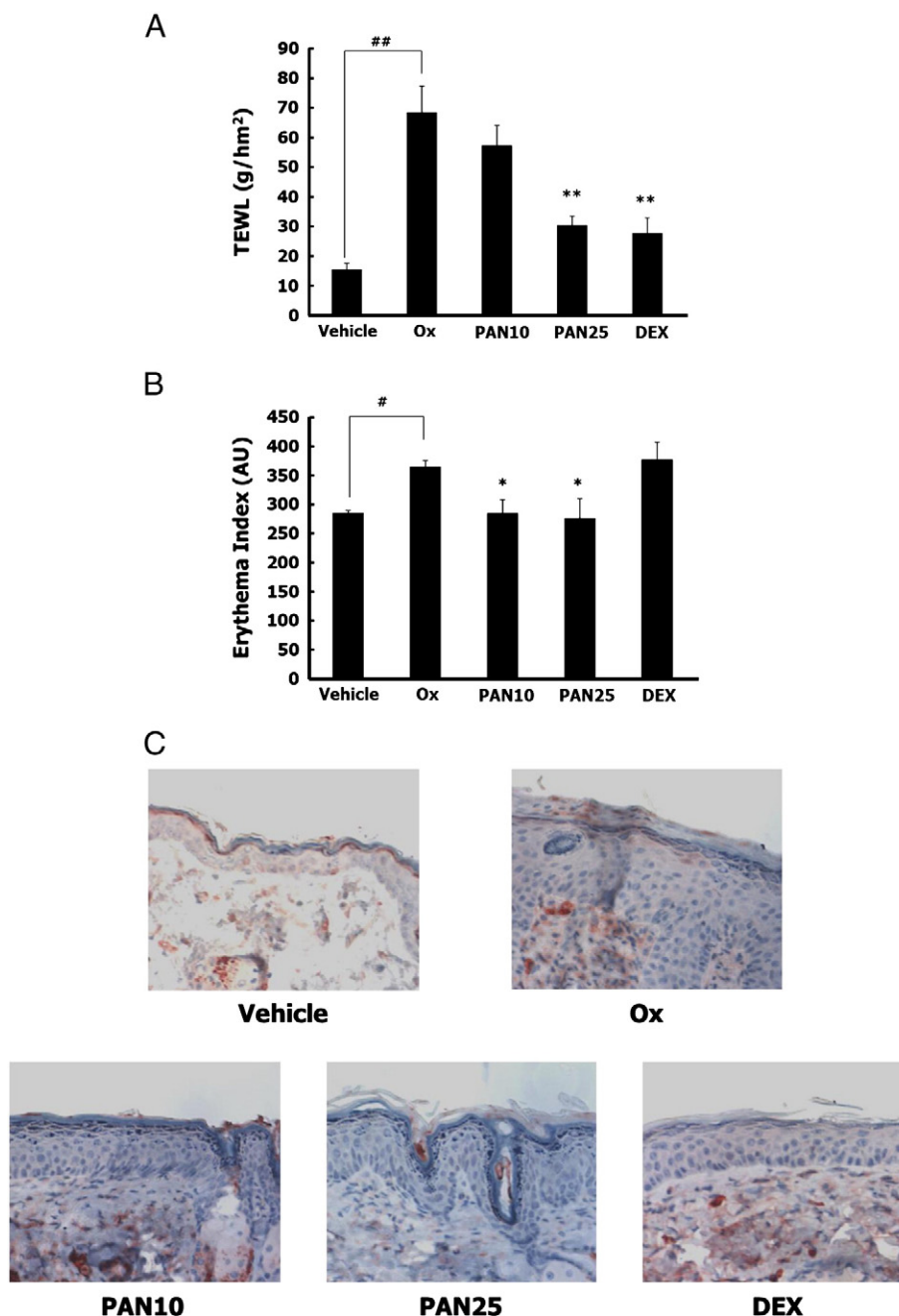


Fig. 4. Effects of panduratin A on TEWL (A), erythema (B), and filaggrin expression (C) of oxazolone-treated hairless mice. The filaggrin expression was analyzed using immunohistochemistry. Results are expressed as mean $\pm$ SD ($n = 5$). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with vehicle group; $^*P < 0.05$ and $^{**}P < 0.01$ compared with oxazolone-treated group.

that the protective effects of panduratin A on skin barrier function alleviate oxazolone-induced skin barrier damage in a hairless mice model.

The effects of panduratin A on the production of IgE and IgG2a in serum

To investigate the effects of panduratin A on allergy-related immunoglobulin, the serum levels of Th2-mediated IgE and Th1-mediated IgG2a were measured. In the oxazolone treatment group, the level of IgE was increased, while that of IgG2a was decreased as compared with vehicle group. However, oral administration of panduratin A alleviated the level of IgE and augmented that of IgG2a as compared with the oxazolone treatment group (Fig. 5A and B). The effects of panduratin A on the IgE and IgG2a levels were comparable to those of DEX. These results indicate that panduratin A may inhibit the development of AD by lowering the level of IgE and increasing that of IgG2a.

The effects of panduratin A on the expression of PPAR α/δ mRNA in the skin

To evaluate the effects of panduratin A on PPAR α/δ expression, the mRNA level of PPAR α/δ in the skin was measured (Fig. 6). Oxazolone treatment reduced expression of PPAR α/δ mRNA. However, panduratin A dose-dependently increased the level of PPAR α/δ mRNA in AD-like skin lesions (Fig. 7). These results indicate that panduratin A elevates PPAR α/δ expression in the skin.

The effects of panduratin A on the mRNA expression of Th1/Th2/Tregs-associated cytokines and transcription factors in the spleen

To assess cytokine and transcription factor production in the spleen, the mRNA expression for Th1/Th2/Treg-associated molecules in the

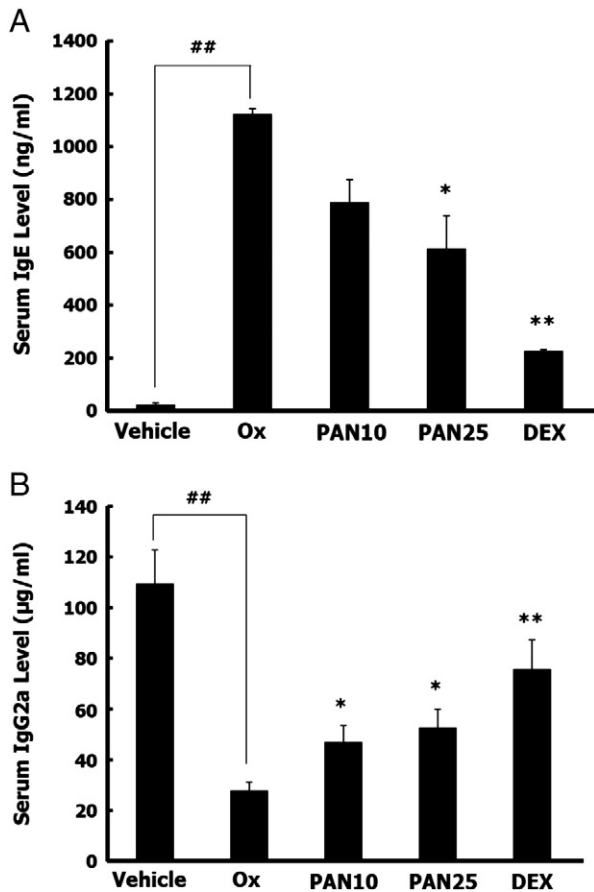


Fig. 5. Effects of panduratin A on production of IgE (A) and IgG2a (B) in serum. Results are expressed as mean $\pm$ SD ($n = 5$). $^{##}P < 0.01$ compared with vehicle group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with oxazolone-treated group.

spleen was measured. Oxazolone treatment decreased mRNA expression of Th1/Treg-associated molecules but increased that of Th2-associated molecules. However, oral administration of panduratin A increased expression of Th1 (IL-12 and T-box family transcription factor; T-bet)/Treg (IL-10, TGF- β , and Foxp3)-associated molecules and decreased that of IL-5, IL-13, and GATA-3, which are Th2-associated molecules (Fig. 8). These results suggest that panduratin A suppresses the Th2-associated molecules and elevates the Th1-associated molecules in the spleen by activating Treg-associated molecules in our AD model.

The effects of panduratin A on the mRNA expression of proinflammatory molecules in the skin

In addition to immunoregulatory cytokines, the mRNA expression of the proinflammatory molecules tumor necrosis factor (TNF)- α , IL-1 β , and protease-activated receptor-2 (PAR-2) was also measured. The mRNA expression of TNF- α , IL-1 β , and PAR-2 was elevated in the skin of the oxazolone-treated group compared with the vehicle group but was decreased in the panduratin A-fed group (Fig. 9). These results suggest that the effect of panduratin A on oxazolone-induced AD model is influenced by TNF- α , IL-1 β , and PAR-2 expression.

As transcription factor nuclear factor-kappa B (NF- κ B) is important in mediating the development of inflammatory responses, mRNA expression of NF- κ B1 and 2 was measured in skin lesions. Oxazolone treatment produced markedly increased expression of both NF- κ B1 and 2 in skin lesions compared with the vehicle group. However, oral administration of panduratin A decreased expression of both NF- κ B1 and 2 in skin lesions (Fig. 9). These results suggest that panduratin A could negatively control the inflammatory responses in skin by suppressing the NF- κ B proinflammatory pathway.

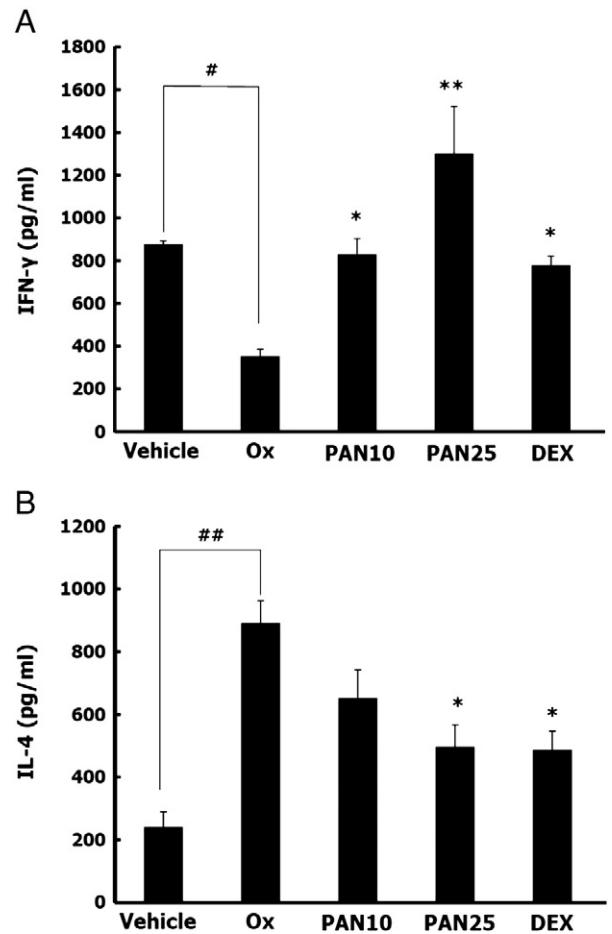


Fig. 6. Effects of panduratin A on production of IFN- γ (A) and IL-4 (B) in serum. Results are expressed as mean $\pm$ SD ($n = 5$). $^{##}P < 0.01$ compared with vehicle group; $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with oxazolone-treated group.

Discussion

Both immunological abnormalities and skin barrier dysfunction contribute to the pathogenesis of AD (Elias et al., 2008), and effective therapy should address both these issues. Treatment with PPAR ligands seems promising because these agents not only have anti-inflammatory activity, but they also show positive effects on cutaneous permeability barrier homeostasis (Fluhr et al., 2009). Previously, a topical PPAR α agonist was shown to inhibit epidermal hyperplasia and inflammatory cell infiltration in a murine model of chronic allergic dermatitis caused by repeated topical application of ovalbumin (Staumont-Salle et al., 2008). Treatment with the PPAR α agonist WY14643 successfully reduced skin expression of IL-4, IL-5, IL-31, IL-1 β , CCL11, and NF- κ B1 and 2 in this model (Staumont-Salle et al., 2008). In addition, Hatano et al. showed that treatment with a PPAR α / δ activator attenuated the clinical dermatitis and, in particular, improved barrier function in another chronic allergic dermatitis murine model induced by repeated topical application of oxazolone. PPAR α / δ activation also reversed the increase in mast cells in the skin of mouse, in addition to decreasing serum IgE and CCL17 levels (Hatano et al., 2010). Studies using PPAR α / δ -selective agonists have shown that PPAR α / δ activation increases the expression of filaggrin, which is important for the formation of the corneous layer and inactivates eosinophil chemotaxis and the NF- κ B pathway (Demerjian et al., 2009; Hatano et al., 2010; Staumont-Salle et al., 2008). Thus, the anti-inflammatory effects of PPAR α / δ agonist application in the skin might be directly derived from a reduction in inflammatory cell infiltration and downregulation of inflammatory cytokines. In our previous study (Kim et al., 2011), panduratin A activated PPAR α / δ expression and activation in L6

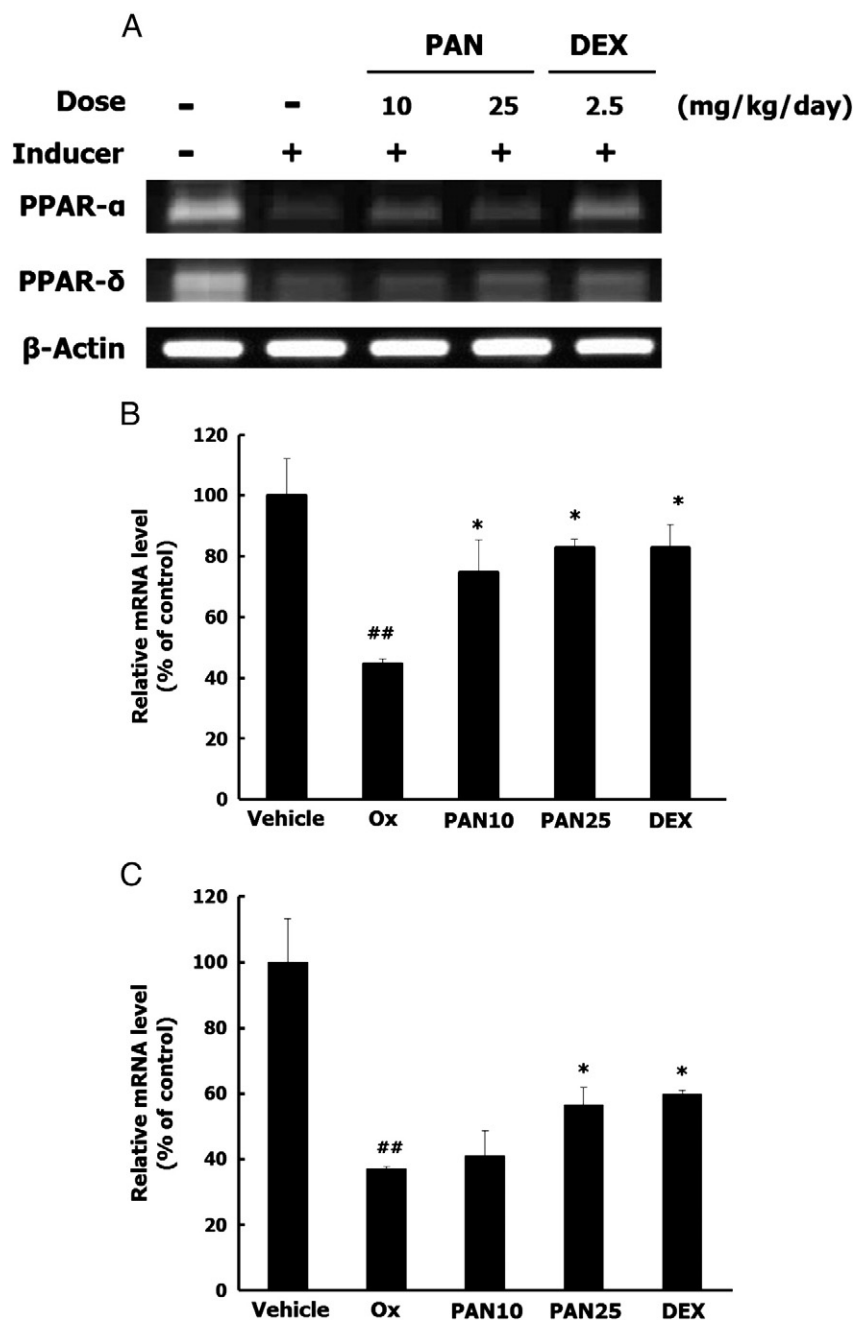


Fig. 7. Effects of panduratin A on PPAR α and PPAR δ expression of oxazolone-treated hairless mice (A–C). Results are expressed as mean $\pm$ SD of three independent experiments. ## p < 0.01 compared with vehicle group; * p < 0.05 compared with oxazolone-treated group.

myoblasts and C57BL/6J mice. For this reason, we investigated whether panduratin A upregulated PPAR α / δ expression and activation in the skin cells and AD-like skin lesions of hairless mice. Interestingly, panduratin A elevated PPAR α / δ and PPRE activation in vitro and increased mRNA expression of PPAR α / δ in vivo (Figs. 2 and 7). Therefore, when considering the results of the various studies that panduratin A has anti-inflammatory activity by inhibiting NF- κ B translocation and pro-inflammatory cytokine expression (Yun et al., 2003; Choi et al., 2012), we suggest that panduratin A suppresses inflammatory response by activating PPAR α / δ .

The skin of patients with AD is characterized by increased TEWL, erythema, infiltration of inflammatory cells, and epidermal thickness (DeBenedetto et al., 2012). Oxazolone, which is a hapten compound, may change the expression of filaggrin, leading to severe deterioration in barrier function (Howell et al., 2009). Thus, we investigated the effects

of panduratin A on TEWL, erythema, infiltration of inflammatory cells, epidermal thickness, and filaggrin expression in oxazolone-sensitized hairless mice. We found that oral administration of panduratin A inhibited TEWL, erythema, infiltration of inflammatory cells, and epidermal thickness and reversed filaggrin expression in oxazolone-sensitized hairless mice (Figs. 3 and 4). These results suggest that panduratin A improves barrier function through stimulation of epidermal differentiation by PPAR α / δ activation.

Development of AD is dependent on the production of cytokines, such as IL-4, IL-5, IL-13, and IL-31, by Th2 cells. This production is transcriptionally regulated by GATA-3, which is a transcription factor of Th2 cells. Because of GATA-3, IgE is mediated by IL-4 and IL-13 (Katayama et al., 2011). On the other hand, the expression of T-bet, which is a transcription factor of Th1 cells, is correlated with the expression of IFN- γ in Th1 cells (Rautajoki et al., 2008). Also, IL-12 exerts its biological effects through

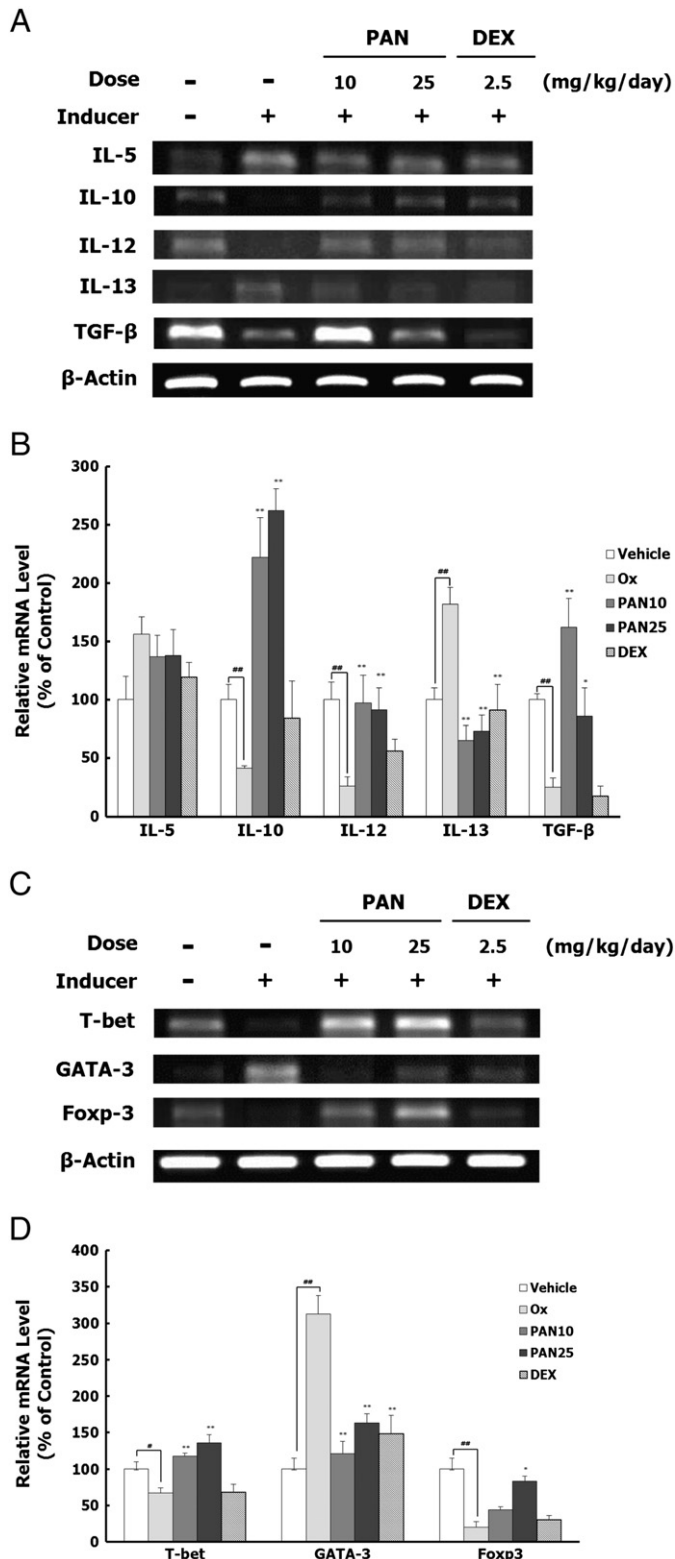


Fig. 8. Effects of panduratin A on mRNA expression of Th1/Th2/Treg-associated (A, B) cytokines and (C, D) transcription factors in the spleen. Results are expressed as mean $\pm$ SD ($n = 5$). * $P < 0.05$ and ** $P < 0.01$ compared with vehicle group; * $P < 0.05$ and ** $P < 0.01$ compared with oxazolone-treated group.

IFN- γ , which is a key cytokine in the downregulation of IgE in vitro and in vivo (Wegmann, 2009). Furthermore, inflammatory cells are recruited to the skin through interactions of chemokines and chemokine receptors. The CC chemokine ligands (CCL) are also considered to be important in the development of skin diseases such as AD (Homey et al., 2007). Oral

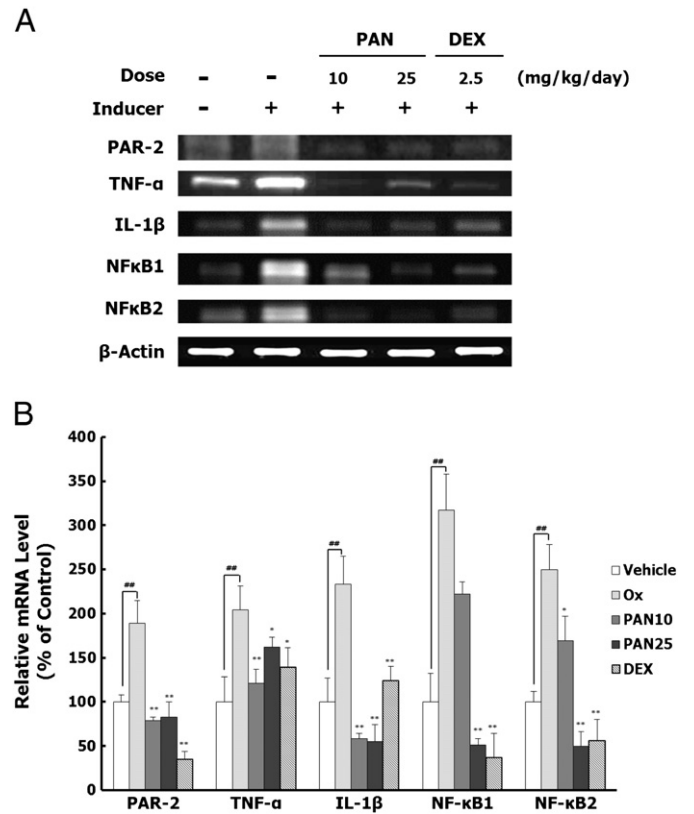


Fig. 9. Effects of panduratin A on mRNA expression of proinflammatory molecules in the skin (A, B). Results are expressed as mean $\pm$ SD ($n = 5$). ** $P < 0.01$ compared with vehicle group; * $P < 0.05$ and ** $P < 0.01$ compared with oxazolone-treated group.

administration of panduratin A resulted in an increase in Th1-associated molecules (IgG2a, IL-12, IFN- γ , and T-bet) in the sera and spleen (Figs. 4B, 5A, and 8). In contrast to its effects on Th1 cytokines in the sera and spleen, panduratin A induced a significant decrease in Th2-mediated molecules (IgE, IL-4, IL-5, IL-13, IL-31, CCLs, CCRs, and GATA-3) in the sera and spleen (Figs. 4A, 5B, and 8). These results indicate that panduratin A may restore the impaired balance between Th1- and Th2-associated immune responses by enhancing the production of Th1 cytokines.

Moreover, panduratin A increased the mRNA levels of IL-10 and TGF- β in the spleen. IL-10 and TGF- β are key mediators in the prevention of allergic inflammation. IL-10 plays an important role in the regulation of immune responses in AD-like skin lesions and the maintenance of peripheral homeostasis (Pickard et al., 2007). In addition, subcutaneous injection of TGF- β was reported to suppress the development of AD-like skin lesions in NC/Nga mice, which was accompanied by the reduction of IgE level in the serum (Sumiyoshi et al., 2002). These reports indicate that IL-10 and TGF- β are important factors in preventing allergic inflammation, and AD-like skin lesions are associated with inhibition of immune-regulatory cytokine expression. Recently, Tregs as key regulators of immunologic development have been reported as important cells in the prevention and treatment of allergic diseases (Akdis and Akdis, 2011). Many papers have shown that Tregs highly express transcription factor Foxp3, which regulates AD (Szegedi et al., 2009). In the present study, the mRNA expression of Foxp3 in the spleen was reduced in the oxazolone-sensitized group, but it was markedly increased by panduratin A (Fig. 8C and D). Thus, Tregs may have had an important role in the tolerance of immune diseases and in ameliorating the inflammation of skin in panduratin A-fed mice.

The production of proinflammatory cytokines, especially TNF- α and IL-1 β in epidermal cells, is one of the fundamental events in AD initiation (Homey et al., 2006). TNF- α and IL-1 β produced at the initiation phase of

AD induce the production of various chemokines that cause the recruitment and infiltration of inflammatory cells in the skin. It was reported that panduratin A exerted anti-inflammatory activity, and TNF- α expression was decreased by panduratin A in mast cells (Choi et al., 2012). Moreover, abnormal expression or activity of serine proteases and PAR-2 has been associated with several inflammatory skin disorders involving barrier abnormalities, including AD (Lee et al., 2010). A recent study reported that SPINK5 knockout mice expressed TSLP in the epidermis at a higher rate than in wild-type epidermis and demonstrated that serine protease directly activates PAR-2, which in turn induces NF- κ B-mediated overexpression of TSLP (Lee et al., 2010). Therefore, we tested if the effect of panduratin A on our oxazolone-induced AD model might be mediated by inhibiting TNF- α , IL-1 β , and PAR-2. In this study, oral administration of panduratin A inhibited the mRNA levels of these markers in skin lesions (Fig. 9).

NF- κ B is activated by several antigens found in inflammatory diseases (Gloire et al., 2006). NF- κ B is a transcription factor that regulates the expression of genes involved in inflammation and apoptosis (Gloire et al., 2006). Activation of NF- κ B plays a pivotal role in inflammatory skin diseases (Aiba et al., 2003). In addition, TNF- α and IL-1 β expression are known to be regulated by NF- κ B activation (Aiba et al., 2003) and PPAR agonists inhibit the activity of NF- κ B (Staumont-Salle et al., 2008). Our results showed that panduratin A suppressed mRNA expression of both NF- κ B1 and 2 in skin lesions (Fig. 9). These results suggest that panduratin A, an activator of PPAR α / δ , could negatively regulate the skin inflammatory responses by inhibiting the NF- κ B proinflammatory pathway.

Conclusion

Taken together, our findings suggest that oral administration of panduratin A to hairless mice with oxazolone-induced AD-like skin lesions has a beneficial effect by improving skin barrier function and modulating a Th1/Th2/Treg-related immune response. Although additional studies are necessary to further characterize the molecular mechanisms underlying the ability of panduratin A to modulate the Th1/Th2/Treg-associated immune response, we anticipate that panduratin A has potential as a therapeutic agent for the treatment of AD.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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