

Pseudoceramide stimulates peroxisome proliferator-activated receptor- α expression in a murine model of atopic dermatitis: molecular basis underlying the anti-inflammatory effect and the preventive effect against steroid-induced barrier impairment

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Abstract Topical pseudoceramides are successfully used in skin barrier repair therapy for atopic dermatitis (AD) and demonstrated to reduce the adverse effects of topical glucocorticoids (GC). However, the molecular mechanisms involved are not fully understood. We investigated whether PC-9S (myristoyl/palmitoyloxostearamide/arachamide MEA, Neopharm, Daejeon, Korea), one of the synthetic pseudoceramides, could stimulate peroxisome proliferator-activated receptor (PPAR) α expression in a hapten [oxazolone (oxa)]-induced AD murine model (oxa-AD mice) and subsequently improved permeability barrier, reduced inflammation, and increased antimicrobial peptides (AMPs) expression. Normal hairless mice and oxa-AD mice were topically treated twice daily with either PC-9S-containing physiologic lipid mixture (PLM), vehicle (PLM), or PPAR α agonist for 4 days. Topical PC-9S significantly increased PPAR α expression in mouse epidermis in vivo and in oxa-AD mice skin comparable with PPAR α agonist. Topical PC-9S-containing PLM significantly reduced basal trans-epidermal water loss (TEWL), surface pH, and mast cell infiltrates and prevented the decline of AMPs expression in oxa-AD mice, which were abrogated by PPAR α antagonist. Then, oxa-AD mice were treated with super-potent topical GC twice daily for 4 days with or without PC-9S co-applications. Co-treatment with PC-9S-containing PLM suppressed GC-induced increase in basal TEWL, epidermal thinning, reduced loricrin expression,

and impaired barrier recovery and these effects were attenuated by PPAR α antagonist. Collectively, our findings suggest that pseudoceramide PC-9S-induced stimulation of PPAR α expression provides a new mechanism by which pseudoceramides show anti-inflammatory property, improve the permeability and antimicrobial barrier function, and prevent the negative effects of topical GC.

Keywords Pseudoceramide · Peroxisome proliferator-activated receptor- α · Glucocorticoid · Atopic dermatitis

Introduction

The epidermal permeability barrier abnormality is an important factor in the development of atopic dermatitis (AD). In addition to a global decrease in stratum corneum (SC) lipids, a selective decrease of ceramides is a characteristic feature of AD and is thought to be associated with disorganization of lamellar bilayer and reduced water holding capacities of SC, leading to dry skin [12, 15]. On the basis of these findings, ceramide-dominant physiologic lipid-based emollients have been tried and found to be effective in AD patients [8, 32].

There are considerable interests in the synthetic pseudoceramides as ingredients of moisturizers because of the potential risk of natural ceramides including inhibition of cellular proliferation and induction of apoptosis and the high cost of skin-identical synthetic ceramides [9]. Pseudoceramides are structurally similar to natural ceramides and have been demonstrated to restore the permeability barrier function after acute or chronic barrier disruption in mice skin [13, 14]. In addition, pseudoceramides are shown to be effective in improving the clinical symptoms and

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barrier function in AD patients [3]. The beneficial effects of topical pseudoceramides for the management of AD are thought to be mediated via the replacement of lipids, which are structurally similar to ceramides; however, the underlying mechanisms are not fully understood.

Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear hormone receptors that exert important function in skin barrier homeostasis by stimulating the epidermal lipid synthesis, formation/secretion of lamellar bodies, and keratinocyte differentiation [20, 22]. In addition, PPARs regulate cutaneous inflammation [24, 29]. PPAR α expression is reduced in the skin of AD patients and of murine AD models and certain activators of PPARs showed a therapeutic benefit in mouse models of AD, suggesting a role of PPAR signaling in AD pathogenesis [11, 28, 30].

In addition to fatty acids and their metabolic products, recent studies have shown that certain sphingolipid analogs activate the transcriptional activities of PPARs in various cell lines [16, 31]. The synthetic cell-permeant C2- and C6-ceramide were demonstrated to increase PPAR δ expression and stimulate ABCA12 expression by a PPAR δ -dependent signaling in keratinocytes [16]. It was also reported that several synthetic sphingolipid analogs have agonistic activities for PPARs in fibroblast cell line NIH3T3 [31]. Based on these observations, we hypothesized that certain pseudoceramides can also stimulate PPAR expression and thereby exert anti-inflammatory and permeability barrier-repairing effects. In this study, we examined the effect of topical synthetic pseudoceramide, PC-9S (myristoyl/palmitoyloxostearamide/arachamide MEA), on the expression of PPAR α in mouse skin in vivo and investigated whether topical PC-9S could restore PPAR α expression in a hapten [oxazolone (oxa)]-induced AD-like mice model (oxa-AD mice). Then, we investigated whether topical PC-9S could normalize the permeability barrier function and reduce inflammation in oxa-AD mice via PPAR α signaling.

Along with the impaired permeability barrier functions, AD patients' skin shows impaired anti-microbial defense system with insufficient induction of antimicrobial peptides (AMPs); therefore, we also investigated the effect of PC-9S in the expression of AMPs in oxa-AD mice.

Topical glucocorticoids (GCs) are the mainstay in the treatment of AD; however, their adverse effects on the structure and function of permeability barrier limit their long-term application. Previous studies have shown that the PPAR α ligand Wy14,643 could prevent the topical GC-induced skin barrier abnormalities [10]. Here, we also investigated whether co-treatment with topical PC-9S could reverse the adverse effects of topical GC on the epidermal structure and function in AD mice model via PPAR α signaling.

Materials and methods

Reagent

WY14,643 (PPAR α activator), GW6471 (PPAR α antagonist), and oxazolone(oxa) were purchased from Sigma Aldrich (Sigma-Aldrich, St. Louis, MI, USA). Primary antibodies, for mouse loricrin and PPAR α , were purchased from ABCam (ABcam, Cambridge, MA, USA). Mouse beta-defensin and CRAMP primary antibodies and secondary antibody, IgG-HRP and IgG-FITC, were purchased from Santa Cruz Biotechnology (Santa Cruz Bio-technology, Santa Cruz, CA, USA). Clobetasol 17-propionate (Dermovate[®]) and clobetasone butyrate (Eumovate[®]) were purchased from GSK (Glaxosmithkline, London, England). PC-9S (myristoyl/palmitoyloxostearamide/arachamide MEA) was supplied by Neopharm (Daejon, South Korea). For in vivo studies, topical formulation of physiologic lipid mixture (PLM) with 0.6 and 1.2 % of PC-9S was prepared (Table 1).

Animals and experimental protocol

Specific pathogen-free female SKH-1 hairless (h/h) mice (6–8 weeks old) were purchased from Orient Bio (Orient Bio Co, Seoul, South Korea) and kept under controlled humidity (50–60 %) and temperature (23–25 °C).

To establish a hapten (oxa)-induced AD-like dermatitis murine model (oxa-AD mice), hairless mice were sensitized by 2 successive days with 60 μ l 1 % oxazolone in acetone. After 1 weeks of 2 consecutive sensitization, 60 μ l of 0.1 % oxazolone was topically applied on both flanks, once every other day for an additional 20 days (total 10th challenge), and then 0.6 or 1.2 % PC-9S-

Table 1 Composition of pseudoceramide PC-9S containing formulation for in vivo study

Pseudo-stratum corneum lipids	
PC-9S	0.6 % and 1.2 %
Stearic acid/cholesterol	1.7 %
Oils and common base	
Cetanol/squalane	5.5 %
Fatty acid triglyceride	3.5 %
Polyols	
Glycerin	5.0 %
1,3-Butylene glycol	5.0 %
Emulsifiers	
Glycerol monostearate	1.5 %
POE (15) glycerylmonostearate	3.0 %
Thickener/carbomer	0.4 %
Water	Up to 100 %

containing topical formulation of physiologic lipid mixture (PLM), PLM alone, or 60 ml of 10 mM WY 14,643 in the vehicle (acetone) was applied twice daily for 4 days. For the antagonist study, PPAR α antagonist GW6471 was topically applied at 10 mM in the vehicle (acetone), 10 min before the treatment with 1.2 % PC-9S-containing PLM.

To investigate the preventive effect of PC-9S-containing topical formulation on the occurrence of adverse effects of topical GC, after treatment of 0.1 % oxa for 20 days (total 10th challenges), clobetasol 17-propionate or clobetasone butyrate was applied twice a day for 4 days from 11th oxa treatment (day 1). Then, from day 2 to day 4, 1.2 % PC-9S containing PLM was treated twice daily to same site for 3 days. For the inhibition study, PPAR α antagonist GW6471 was topically applied at 10 mM in the vehicle (acetone), 10 min before the treatment with 1.2 % PC-9S-containing PLM. At the end of the treatment period, trans-epidermal water loss (TEWL) and barrier recovery rate were measured and skin biopsies were taken for further analysis.

Immunohistochemistry

Skin biopsies were taken from oxa-AD mice for histological analysis of epidermal expressions of loricrin and PPAR α . Paraffin-embedded sections were cut into 5- μ m and deparaffinized in xylene and rehydrated with sequential treatments of graded ethanol solution. After blocking the non-specific antibody binding by incubation with a serum free protein (Dako, Carpinteria, USA) for 15 min at room temperature, sections were incubated with primary antibodies (1:100 dilution) at 37 °C for 30 min, and with secondary antibodies for 30 min at room temperature. Sections were then drenched with a horseradish peroxidase (HRP)-conjugated secondary antibody for 30 min at room temperature and washed with tap water. Stained sections were visualized by microscopy equipped with digital camera.

Real-time quantitative reverse transcription polymerase chain reaction

Tissue samples were homogenized in TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA), and total cellular RNA was extracted from tissue samples or cultured cells. RNA was reverse transcribed and the resulting cDNA was synthesized from 500 ng total RNA using TaKaRa RNA PCR Kit Ver.2.1 (TaKaRa BIO INC. Shiga, Japan). To perform real-time quantitative PCR, primers were designed using NCBI primer-blast. The following primers were used: β -actin (F:5'-GATGAGATTGGCATGGCTTT-3' and R:5'-CACCTTCACCGTTCCAGTTT-3'), PPAR α

(F:5'-CCTCAGGGTACCACTACGGAGT-3' and R:5'-GCCGAATAGTTCGCCGAA-3'). The detection of amplification was performed using an Express SYBR Green ER Supermix with premixed ROX (Invitrogen Life Technologies, Carlsbad, CA, USA), power SYBR green PCR master mix (Applied Biosystems) and an ABI 7300 (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. The reaction condition was set to 95 °C for 10 min and then 95 °C for 10 s, 40 cycles of 60 °C for 1 min.

Western blot

For western blotting of PPAR α , mBD3, and CRAMP, the peptides were extracted from epidermis separated from full-thickness mouse skin. The proteins were separated by 4–12 % SDS-PAGE with 3[*N*-morpholino]propanesulfonic acid (MOPS) buffer (Invitrogen) and transferred to polyvinylidene difluoride membranes. After blocking with 5 % non-fat dry milk, membranes were incubated overnight at 4 °C with anti-PPAR α , anti-mBD3, or anti-CRAMP antibodies (Santa Cruz Bio-technology, Santa Cruz, CA, USA) diluted 1:200. After several washes, peroxidase-conjugated anti-goat antibody was added (final dilution 1:2000) for 1 h at room temperature. Signals were detected using the ECL Plus Western blotting Detection System (Amersham, Buckinghamshire, UK).

Immunofluorescence staining

For staining of mouse beta-defensin-3 (mBD-3) and cathelicidin murine-related antimicrobial peptide (CRAMP), skin biopsies and paraffin blocks were prepared. Sections were wetted with primary antibody (1:100 dilution) at 37 °C for 30 min and FITC-conjugated secondary antibodies were incubated at room temperature for 30 min. After 3 washes with TBS buffer, PI (1:1000 dilution) was performed at room temperature for 3 min. Stained sections were visualized by microscopy equipped with digital camera.

Mast cell counting

For mast cell staining, paraffin-embedded sections were stained with potassium permanganate solution for 2 min and rinsed with distilled water. Then, slides were incubated in potassium metabisulphite solution for 1 min or until sections appeared white. After washing with tap water for 3 min, the slides were immersed into the acidified toluidine blue solution for 5 min. After washing slides in tap water, slides were observed by light microscopy equipped with digital camera.

Skin functional measurements

TEWL was measured with TewameterTM300 (Courage + Khazaka electronic GmbH, Köln, Germany) at day 3 from 11th oxa treatment (day 1). For barrier recovery experiments, repeated tape stripping was performed on both flanks of hairless mice and TEWL was measured at the same sites at 3, 6 and 48 h after tape stripping. The barrier recovery results are expressed as percentage recovery, which was calculated by applying the following formula: $[(\text{TEWL immediately after barrier disruption} - \text{TEWL at indicated time point}) / (\text{TEWL immediately after barrier disruption} - \text{baseline TEWL})] \times 100 \%$.

Statistical analysis

Otherwise noted, the results are expressed as mean $\pm$ standard deviation (SD). *p* values less than 0.05 were considered as statistically significant. Either two-tailed Student *t* test or one-way ANOVA with Dunnett's post test was performed using Graph Pad In Stat version 3.00 software (Graph Pad Software, San Diego, CA, USA).

Results

Pseudoceramide PC-9S increases PPAR α expression in normal mouse skin and oxa-AD mice skin

First, we investigated whether pseudoceramide PC-9S regulates PPAR α expression in mouse skin *in vivo*. As shown in Fig. 1a, topical treatment with PPAR α activator Wy-14,643 significantly increased PPAR α protein expression in hairless mouse epidermis, suggesting that PPAR α activator not only activates the already-existed PPAR α , but also stimulates the expression of PPAR α , which might further contribute to the beneficial effects of PPAR α activation. Similarly, topical application of either 0.6 or 1.2 % of PC-9S-containing PLM significantly upregulated PPAR α protein expression compared to that of PLM (vehicle) alone-treated skin (Fig. 1a).

We next studied whether pseudoceramide PC-9S can restore PPAR α expression in a hapten (oxa)-induced AD-like dermatitis murine model. Consistent with the previous studies [4, 30], the epidermal PPAR α mRNA and protein levels were significantly reduced in oxa-induced AD-like skin lesions of hairless mice compared with those of the untreated control mice skin by immunohistochemistry, western blot, and real-time PCR (Fig. 1b–d). Topical treatment of oxa-AD mice with either 0.6 or 1.2 % of PC-9S-containing PLM significantly increased mRNA and protein expression of PPAR α in a concentration-dependent manner compared to that of PLM alone-treated sites

(Fig. 1b–d). Wy-14,643 treatment also significantly increased PPAR α mRNA and protein expression in oxa-AD mice skin (Fig. 1b–d). These findings suggest that pseudoceramide PC-9S can restore and further increase PPAR α expression in the epidermis of AD mouse model.

Pseudoceramide PC-9S-containing PLM normalizes the permeability barrier function in oxa-AD mice and PPAR α antagonist treatment attenuated the effects of PC-9S

On the basis of our results demonstrating that PC-9S stimulates PPAR α expression, we next investigated whether topical pseudoceramide PC-9S-containing PLM could normalize the permeability barrier function in oxa-AD mice via PPAR α signaling. As previously reported [11], topical Wy-14,643, a known PPAR α activator, normalized the permeability barrier function by significantly reducing basal TEWL and surface pH in oxa-AD mice. Then, we observed a similar improvement of barrier function in PC-9S-containing PLM-treated group. Increase of TEWL by repeated oxa treatment was significantly prevented by topical application of either 0.6 or 1.2 % of PC-9S-containing PLM, while PLM alone did not significantly reduce TEWL compared to that of vehicle-treated sites (Fig. 2a). SC hydration was not significantly changed after treatment with either 0.6 or 1.2 % of PC-9S-containing PLM (data not shown). Only 1.2 % of PC-9S-containing PLM significantly prevented the increase of skin surface pH by oxa treatment (Fig. 2b). These results suggest that PC-9S can normalize permeability barrier function in AD mice model. To determine whether a PPAR α signaling pathway contributes to the PC-9S-induced permeability barrier repair, we performed antagonist experiments. Pretreatment with PPAR α antagonist (GW6471, 10 mM) significantly attenuated the inhibitory effect of 1.2 % of PC-9S-containing PLM on the increase of basal TEWL and skin surface pH by repeated oxa treatment (Fig. 2). These results suggest that PPAR α signaling partly mediates pseudoceramide PC-9S-induced beneficial effects on the permeability barrier function in AD mice model.

Pseudoceramide PC-9S-containing PLM reduces inflammation in oxa-AD mice and PPAR α antagonist treatment attenuated the anti-inflammatory effects of PC-9S

To further investigate the anti-inflammatory activity of PC-9S on oxa-AD mice, skin biopsies were taken and histological observation was performed. PLM alone or PC-9S-containing PLM significantly reduced the total number of inflammatory cells compared to that of vehicle-treated

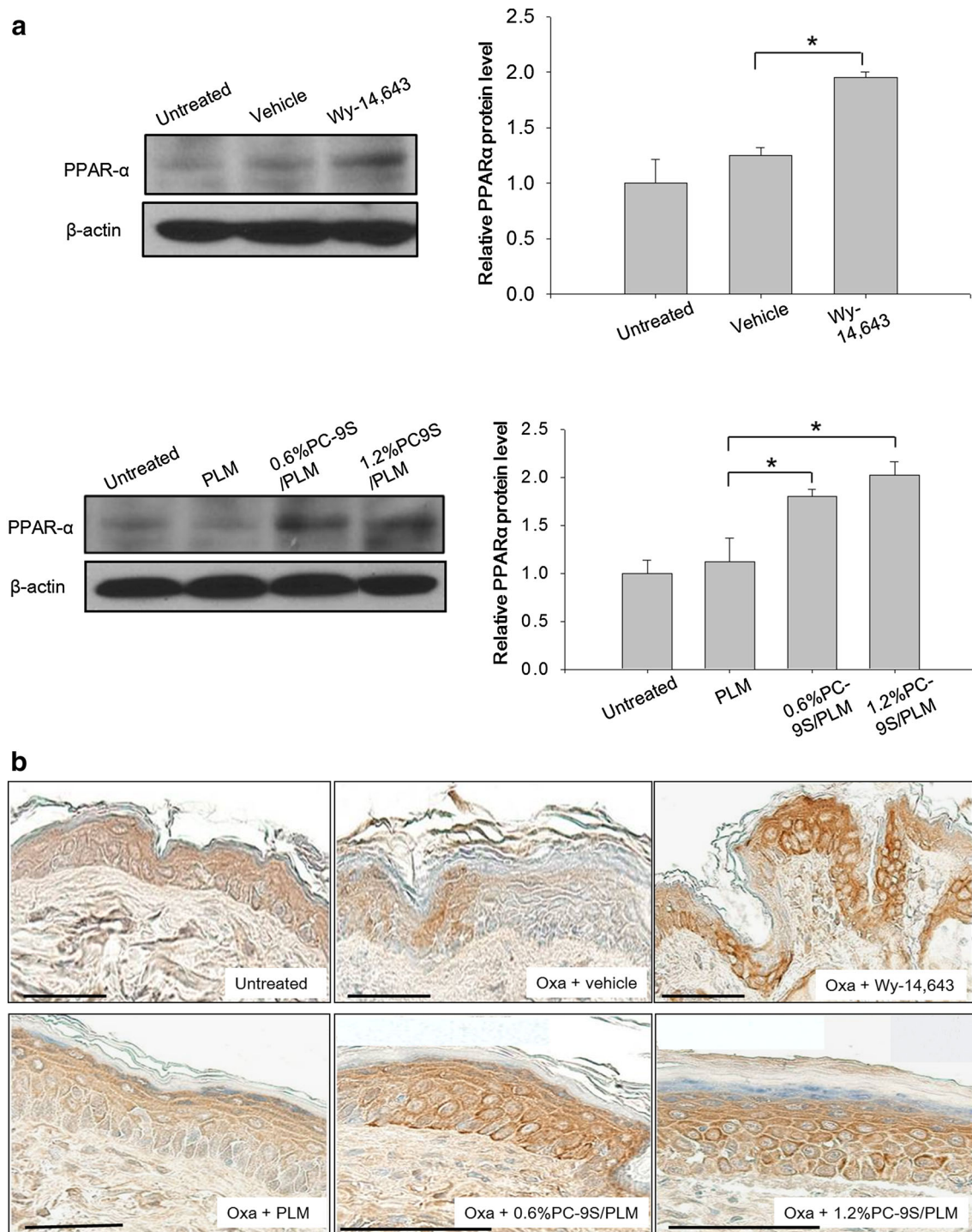


Fig. 1 PPAR α expression is upregulated by pseudoceramide PC-9S-containing physiologic lipid mixture (PLM) in normal mouse skin (**a**) and oxa-AD mice model (**b–d**). Normal hairless mouse and oxa-AD mice were treated with 0.6 or 1.2 % PC-9S-containing topical formulation of PLM or 10 mM Wy-14,643 in the vehicle (acetone) for 4 days. **a, c** PPAR α protein expression was evaluated by western blotting analysis ($n = 3$ mice per group). Representative western blot images and scanning densitometry of protein expression are shown. **b** The protein expression of PPAR α was assessed by

immunohistochemistry on oxa-AD mice skin treated with PC-9S-containing PLM or Wy-14,643. Data shown are the representative of six different mice per each treated group. Scale bar 100 μ m. **d** Relative gene expression of PPAR α was analyzed by quantitative real-time PCR. Values in **a, c, d** are shown as mean $\pm$ SD for three independent experiments. * $p < 0.05$ vs. untreated group; + $p < 0.05$ and ++ $p < 0.01$ vs. vehicle (acetone) treated group; # $p < 0.05$ and ## $p < 0.01$ vs. PLM-only treated group

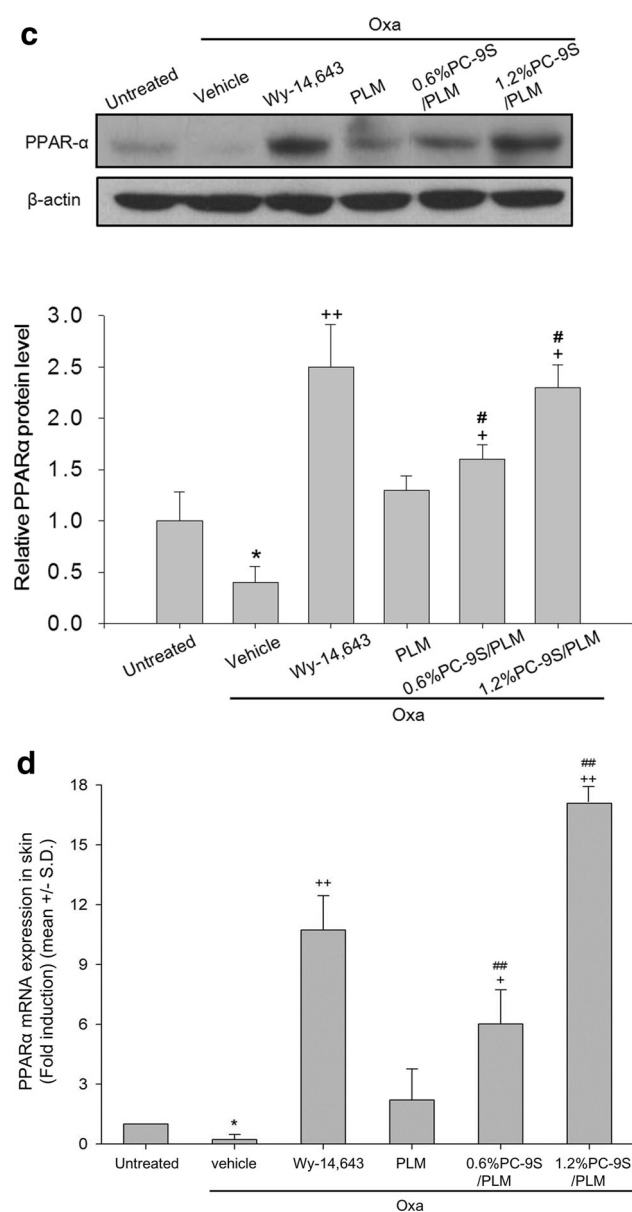


Fig. 1 continued

sites, but the decrease in inflammatory infiltrates was largest in 1.2 % of PC-9S-containing PLM treated sites (Fig. 3a). The mast cell count was also significantly decreased in PC-9S-containing PLM-treated sites in a concentration-dependent manner; however, there was no significant difference between PLM alone and vehicle-treated sites (Fig. 3b). Wy-14,643 also significantly inhibited the oxa-induced inflammation (Fig. 3). Pretreatment with PPAR α antagonist (GW6471, 10 mM) significantly inhibited the 1.2 % of PC-9S-containing PLM-induced reduction in the infiltrates of total inflammatory cells and mast cells in oxa-AD mice (Fig. 3). These findings suggest that PC-9S-containing PLM exerts anti-inflammatory

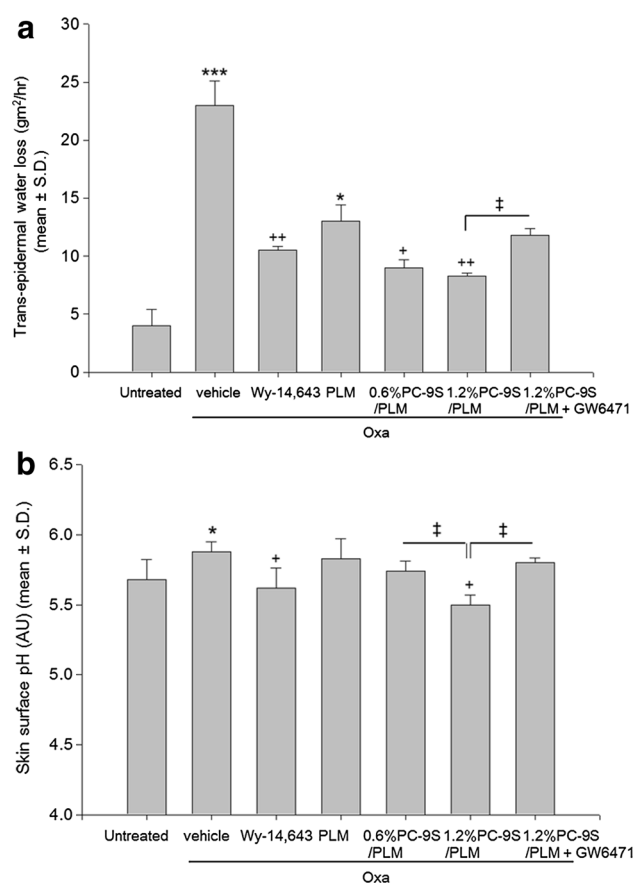


Fig. 2 Normal topical treatment of oxa-AD mice with pseudoceramide PC-9S-containing PLM results in a significant reduction of basal TEWL and skin surface pH, both of which were abrogated by PPAR α antagonist. Basal TEWL (**a**) and skin surface pH (**b**) were measured using Tewameter and skin pH meter, respectively, 48 h after the final application (12th challenge) with oxazolone (day 5). Mean values of five measurements per animal were determined and *n* was 6 for all the experiment. **p* < 0.05 and ****p* < 0.001 vs. untreated group; +*p* < 0.05 and ++*p* < 0.01 vs. vehicle (acetone) treated group; ⁺*p* < 0.05

effects comparable to the known PPAR α activator and the anti-inflammatory effects of PC-9S-containing PLM are partly mediated by PPAR α signaling pathway.

Pseudoceramide PC-9S-containing PLM prevents the decrease of antimicrobial peptides expression in oxa-AD mice and PPAR α antagonist treatment attenuated the effects of PC-9S

Impaired antimicrobial barrier is a characteristic feature in AD and it was reported that repeated treatment of oxa induced downregulation of epidermal expression of AMPs, which might be resulted from the Th2 cytokines induced by oxa treatment [23]. We, therefore, examined whether topical PC-9S or PPAR α activator can restore the expression of AMPs expression in oxa-AD mice.

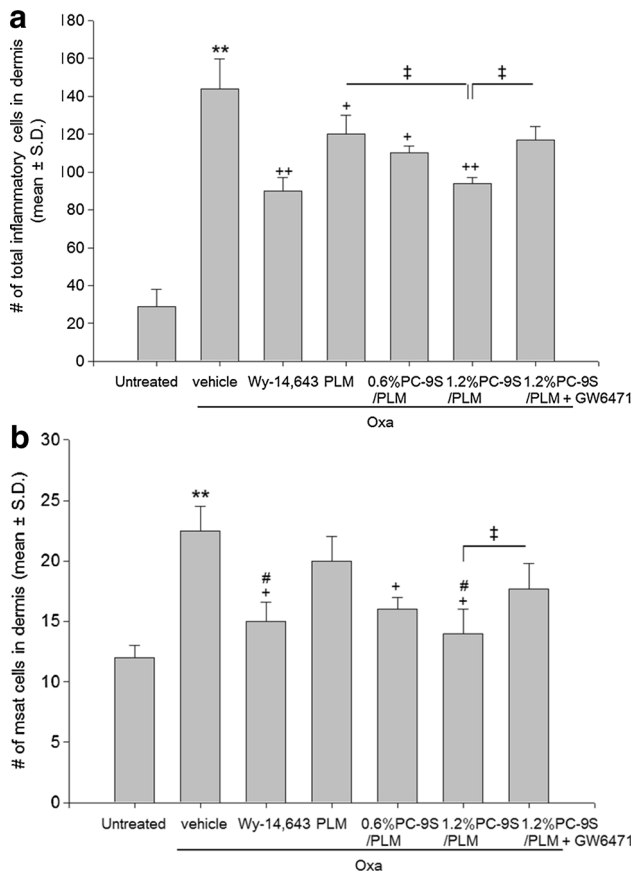


Fig. 3 Topical treatment of oxa-AD mice with pseudoceramide PC-9S-containing PLM results in a significant reduction of inflammatory cell infiltrates, which was inhibited by PPAR α antagonist. The number of total inflammatory cells in dermis per high power field was assessed by H&E staining on the biopsy samples (**a**). Mast cells were quantified as toluidine blue-positive cells and expressed as mast cells/mm² (**b**). ** $p < 0.01$ vs. untreated group; + $p < 0.05$ and ++ $p < 0.01$ vs. vehicle (acetone) treated group; # $p < 0.05$ vs. PLM-only treated group; ‡ $p < 0.05$

The expression of cathelin-related antimicrobial peptide C (CRAMP) and mouse beta-defensin 3 mouse beta-defensin (mBD3) was assessed by immunohistochemical staining and western blotting. As shown in Fig. 4, repeated treatment of oxa downregulated both AMPs expression. The western blot analysis revealed that topical PC-9S-containing PLM significantly increased both AMPs expression compared with that of PLM-only treated sites, similar to that seen in PPAR α activator-treated sites (Fig. 4c). In addition, pretreatment with PPAR α antagonist (GW6471, 10 mM) significantly attenuated the induction of CRAMP and mBD3 expression by 1.2 % of PC-9S-containing PLM treatment in oxa-AD mice (Fig. 4c). These results suggest the involvement of PPAR α signaling in pseudoceramide PC-9S-containing PLM-induced restoration of AMPs expression in AD mice model.

The combined treatment of PC-9S-containing physiological lipid mixtures and GC normalizes the topical GC-induced impairment in permeability barrier function and structure in oxa-AD mice via PPAR α signaling

Previously, we reported that PLM containing pseudoceramide can prevent the topical GC-induced permeability barrier dysfunction in mice ear skin [19]. To investigate a new mechanism through which pseudoceramide exerts the preventive effect on the topical GC-induced barrier impairment, we investigated whether PC-9S-containing PLM can reduce the adverse effect of topical GC on skin barrier function in a mouse model of AD via stimulation of PPAR α . Either super-potent [clobetasol 17-prolionate (CP)] or moderate-potent GC [clobetasone butyrate (CB)] was applied to oxa-AD mice for 4 days from 11th oxa treatment (day 1). Then, from day 2 to day 4, 1.2 % PC-9S containing PLM was treated twice daily to the same site for 3 days. Interestingly, changes of basal TEWL were different between the CP- and CB-treated sites. While CP treatment significantly increased the TEWL value, topical application of less-potent GC (CB) slightly, but not significantly, reduced the TEWL value (Fig. 5a). Based on these findings, we suggest that while topical GC can alleviate the inflammation and result in an improved barrier function, temporary aggravation of permeability barrier function, as adverse effects, can be observed for the highest potency GC treatment. Sequential treatment of PC-9S-containing PLM after GC significantly decreased the basal TEWL, compared to the GC-only treated sites, indicating the preventive effects of PC-9S-containing PLM for the GC-induced barrier dysfunction (Fig. 5a). We also showed that topical treatment with GC alone or the sequential treatment of GC and PC-9S similarly reduced the inflammatory infiltrates compared with those of vehicle-treated group (Fig. 5c). In addition, barrier recovery rate was significantly higher in oxa-AD mice treated with the combination of GC and PC-9S than in oxa-AD mice treated with GC alone at 3, 6, and 48 h (Fig. 5b). Disrupted epidermal differentiation and skin atrophic changes are well-known adverse effects of topical GC. As expected, oxa-AD mice showed marked epidermal thickening and topical GC alone or GC combined with PC-9S treatment normalized epidermal hyperplasia (Fig. 5d). However, 4 days treatment with super-potent GC (CP) alone induced significant thinning of epidermis, while the sequential co-treatment with PC-9S-containing PLM prevented the CP-induced epidermal atrophic changes. We also investigated the effects of PC-9S on the topical GC-induced downregulation of differentiation marker expression. Co-treatment of PC-9S-containing PLM with super-potent GC normalized and even increased the expression of loricrin in oxa-AD

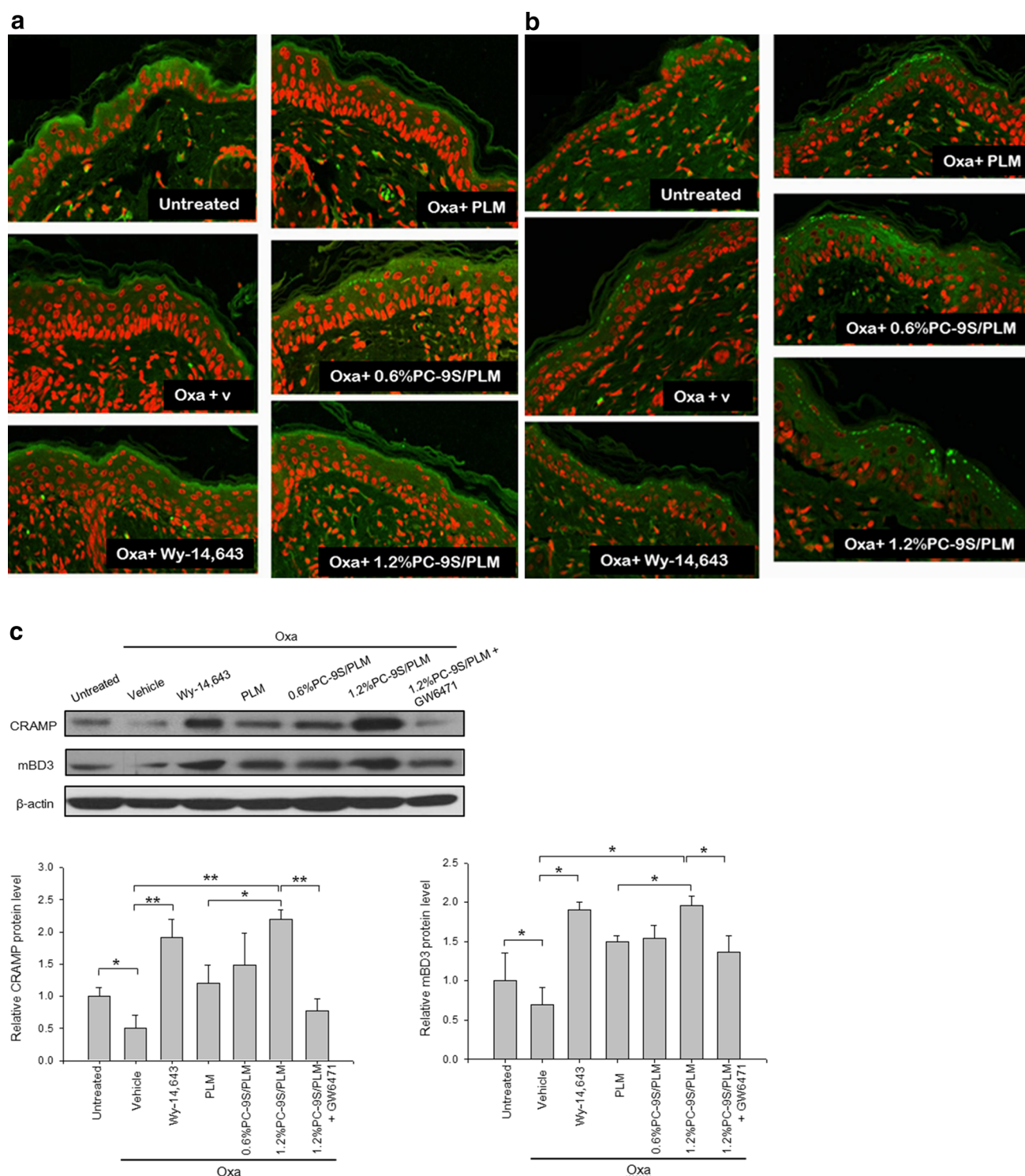


Fig. 4 Pseudoceramide PC-9S-containing physiologic lipid mixture (PLM) increases antimicrobial peptides expression in oxa-AD mice model and PPAR α antagonist attenuated PC-9S-induced expression of antimicrobial peptides. The protein expression of CRAMP (a) and mBD3 (b) was assessed by immunohistochemistry on oxa-AD mice skin treated with PC-9S-containing PLM or PPAR α agonist Wy-14,643 as described in Methods. Data shown are the representative of six different mice per each treated group. Magnifications $\times 200$.

c Western blot of CRAMP and mBD3 expression in lysates from the epidermis of untreated control, oxa-AD mice treated with vehicle, PPAR α agonist Wy-14,643, PLM, and PC-9S-containing PLM with or without PPAR α antagonist GW6471. Semiquantitative densitometric analysis of digitized blots given as mean CRAMP or mBD3/ β -actin signal $\pm$ SD ($n = 3$), $*p < 0.05$ and $**p < 0.01$ by Student's t test

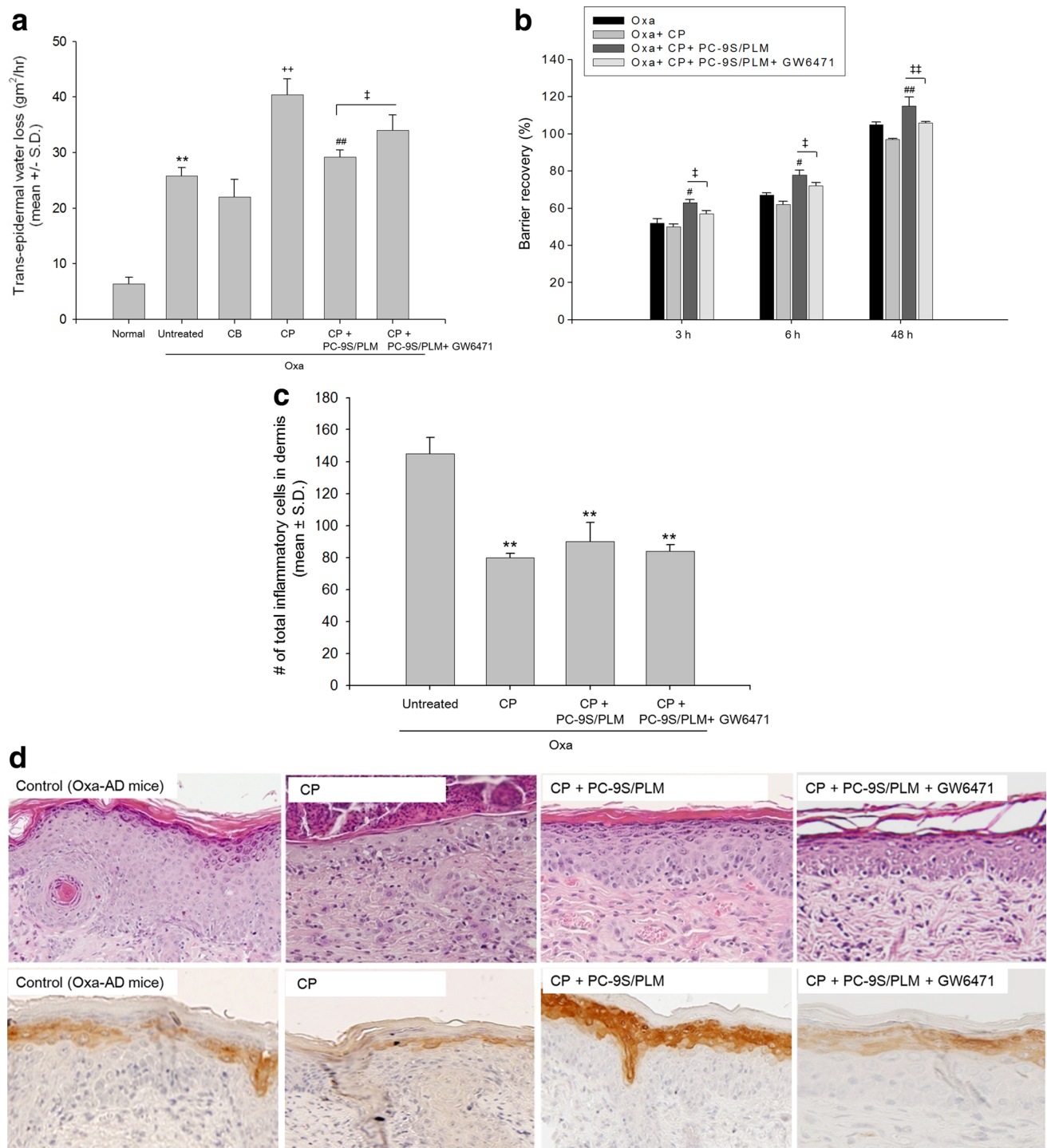


Fig. 5 Pseudoceramide PC-9S-containing PLM normalizes the basal TEWL and barrier recovery after potent topical GC treatment and prevents the topical GC-induced epidermal thinning and downregulation of loricrin expression in oxa-AD mice via PPAR α signaling. **a** TEWL was measured using Tewameter at day 3 from 11th oxa treatment (day 1). **b** After 4 days of topical GC or GC plus PC-9S-containing PLM with or without PPAR α antagonist GW6471, repeated tape stripping was performed on both flanks of hairless mice and the barrier function was evaluated by measuring the TEWL value. Barrier recovery rate was calculated after 3, 6, and 48 h of barrier disruption. The number of total inflammatory cells in dermis

per high power field was assessed by H&E staining on the biopsy samples (**c**). ** p < 0.01 vs. normal group; ++ p < 0.01 vs. untreated group; # p < 0.05 and ## p < 0.01 vs. CP only treated group; † p < 0.05 and ‡ p < 0.01. **d** H&E staining and immunohistochemical staining of loricrin in oxa-AD mice skin treated with topical GC or GC plus PC-9S-containing PLM with or without PPAR α antagonist GW6471 were performed as described in “Methods”. Data shown are the representative of six different mice per each treated group. Magnifications $\times 400$. CP clobetasol 17-prolionate, CB clobetasone butyrate; PC-9S/PLM: 1.2 % PC-9S-containing physiological lipid mixture

mice, suggesting the stimulating effects of PC-9S on the epidermal differentiation (Fig. 5d).

Then, we investigated whether a PPAR α signaling pathway contributes to the preventive effect of PC-9S-containing PLM on the super-potent GC-induced adverse effects on the function and structure of permeability barrier in AD mice model. Pretreatment with PPAR α antagonist (GW6471, 10 mM) was performed 30 min prior to PC-9S-containing PLM treatment for 3 days. PPAR α antagonist attenuated the inhibitory effects of PC-9S-containing PLM on the potent GC-induced increase in basal TEWL and decrease in barrier recovery rate (Fig. 5a, b). In addition, the preventive effect of PC-9S-containing PLM on the potent GC-induced epidermal thinning and downregulation of loricrin expression was attenuated by the application of PPAR α antagonist. Taken together, these data suggest that the preventive effect of PC-9S-containing PLM on the super-potent GC-induced adverse effects on permeability barrier was caused, at least in part, by the PPAR α signaling pathway.

Discussion

Pseudoceramides have similar molecular properties to ceramides and there have been many clinical studies on the effectiveness of moisturizers containing pseudoceramides in the management of AD patients. Moreover, pseudoceramides have been proved to have anti-inflammatory effect both in vitro and in vivo and be beneficial in AD mice model [17, 18]. However, the mechanism of action of pseudoceramide has not been fully elucidated. In this study, we demonstrated that pseudoceramide PC-9S-containing topical formulation of PLM normalized and further increased PPAR α expression at mRNA and protein levels in a murine model of AD. Although we cannot rule out that the other lipid components of PLM as a vehicle of PC-9S, i.e., fatty acid triglycerides and stearic acid, might also have PPAR activating property; however, PPAR α expression was significantly upregulated by PC-9S-containing PLM compared to that by PLM alone, in a concentration-dependent manner, indicating the PPAR α stimulating activity of PC-9S.

The expression of PPAR α was reported to be reduced in the lesional skin of AD; however, whether this downregulation is the primary pathogenic mechanism or a consequence of inflammation is yet to be determined. Previous studies demonstrated that PPAR α expression could be regulated by cytokine milieu. PPAR α expression in keratinocytes was demonstrated to be induced by TNF α and IFN γ , whereas, IL-4 was found to inhibit the upregulation of PPAR α [2]. Chiba et al. [4] recently reported that PPAR α expression was reduced in the inflamed skin of

murine AD model (NC/Nga mice) and topical treatment with PPAR α agonist or GC normalized the expression of PPAR α along with amelioration of the cutaneous inflammation, suggesting that inflammation in atopic eczema might alter the PPAR α expression. In addition, several studies showed that PPAR α expression was downregulated by permeability barrier abrogation using human living skin equivalents and irritant contact dermatitis murine model, suggesting that PPAR α expression could be regulated by altered barrier structure or function [2]. In this study, we found that topical PC-9S-containing PLM significantly reduced the number of total inflammatory cells and mast cell density compared to the PLM alone group in oxa-AD mice. PC-9S-containing PLM, but not the PLM alone, also normalized the increased TEWL and surface pH induced by repeated challenge of hapten. Taken together, we cannot exclude the possibility that the reduced inflammation and normalized barrier function by topical pseudoceramide could contribute to the enhanced expression of PPAR α in AD mice model. However, in this study, we clearly demonstrated that PC-9S-containing PLM significantly upregulates the PPAR α protein expression in the epidermis of hairless mouse skin in vivo compared with that of the PLM alone-treated skin, suggesting that PC-9S can directly upregulate the expression of PPAR α in keratinocytes.

Moreover, we showed that topical PC-9S significantly improved permeability barrier function and reduced inflammation in oxa-AD mice, to an extent comparable to PPAR α activator. Both the permeability barrier-repairing effect and the anti-inflammatory effect of PC-9S in AD mice model were significantly attenuated by PPAR α antagonist application. Taken together, these findings suggest that pseudoceramide PC-9S can stimulate PPAR α expression and subsequently improved permeability barrier function and exhibit potent anti-inflammatory activity in a murine model of AD, at least in part, by PPAR α signaling.

In addition to the restoration of permeability barrier, the restoration of antimicrobial barrier is another important strategy for the management of AD. The high susceptibility to cutaneous infections in AD patients is associated with insufficient expression of AMPs [25, 26]. Thus, we further investigated the effect of PC-9S on the expression of AMPs in AD mice model. Similar to human AD, oxa-AD mice displayed decreased expression of CRAMP and mBD3 and topical PC-9S-containing PLM significantly increased the expression of AMPs in oxa-AD mice. Treatment with PPAR α activator restored the AMPs expression in oxa-AD mice. Furthermore, PC-9S-induced restoration of the epidermal protein levels for both CRAMP and mBD3 in an animal model of AD was attenuated by PPAR α antagonist pre-treatment. Therefore, we suggest that the increase in AMPs expression induced by PC-9S is partly mediated by PPAR α stimulation. In addition, these results suggest that

pseudoceramide PC-9S can contribute to the improvement of the cutaneous anti-microbial barrier functions, as well as permeability barrier function in AD.

Permeability barrier and antimicrobial barrier were demonstrated to be co-regulated and the relationship between these two barriers is thought to be mediated by AMPs [1, 6]. Given our results showing the beneficial effect of PC-9S or PPAR α activator on the permeability barrier function, we can postulate that the restoration of AMPs after PC-9S or PPAR α activator treatment may be partly due to the improved permeability barrier homeostasis. However, it is still possible that PPAR α can be directly involved in the increase of AMPs expression. Previous study showed that PPAR γ directly regulated β -defensin 1 in human colonocytes and mediated antimicrobial immunity in the colon [27]. In keratinocytes, PPAR γ has been shown to regulate the 1,25D₃-induced human beta-defensin-3 and cathelicidin expression by regulating AP-1 and p38 activity [5]. Future studies will be necessary to determine that PPAR α directly mediates the PC-9S-induced AMPs expression.

Several studies proved that co-treatment of pseudoceramide containing PLM reduced the topical GC-induced adverse effects on skin barrier in acute and chronic dermatitis murine models [18, 20, 21]. In this study, we also found that the sequential combination of super-potent GC and PC-9S-containing PLM could reverse the adverse effects of GC on the permeability barrier function and epidermal differentiation. The preventive effect of PC-9S on the potent GC-induced structural and functional abnormalities in permeability barrier was significantly blunted by PPAR α antagonist, suggesting that pseudoceramide PC-9S upregulates PPAR α expression, leading to restore barrier dysfunction caused by super-potent GC.

Topical treatment with mid-potent GC has been shown to improve the AD-like skin lesion and barrier impairment by suppressing inflammation [33]. In line with this, Chiba et al. [4] demonstrated that topical GC could reverse PPAR α expression in NC/Nga AD-like mice skin, accompanied with amelioration of inflammation. In the present study, we also observed that mid-potent GC (CB) decreased TEWL and reduced epidermal thinning in oxa-AD mice. In contrast, we found that super-potent GC (CP) induced increased TEWL, reduced barrier recovery rate, and skin atrophy in our AD mice model, suggesting that despite reduced inflammation, super-potent GC can aggravate skin barrier structure and function. In addition, we confirmed that pseudoceramide PC-9S can upregulate PPAR α expression not only by suppressing inflammation but also by direct stimulation of keratinocytes in normal hairless mouse skin in vivo.

Based on our findings and the prior studies which showed that PPAR α activator prevented the barrier

impairment induced by topical GC [7, 10], we suggest that the PPAR α stimulating effect of pseudoceramide PC-9S is a new mechanism to explain the beneficial effect of pseudoceramide on preventing potent GC-induced side effects in AD mice model.

In summary, our results demonstrate that pseudoceramide PC-9S stimulates PPAR α expression in mouse skin in vivo. Pseudoceramide PC-9S-containing topical formulation of PLM can restore and further increase the expression of PPAR α in an animal model of AD and normalized the permeability and antimicrobial barrier and decreased inflammation in AD mice model via PPAR α signaling. In addition, we demonstrate that combined treatment with pseudoceramide PC-9S and super-potent GC significantly reduced the topical GC-induced structural and functional abnormalities in permeability barrier in AD mice skin, at least in part, via PPAR α signaling. Taken together, we suggest that pseudoceramide PC-9S-induced stimulation of PPAR α expression provides a new mechanism by which pseudoceramides show anti-inflammatory property, improve the permeability and antimicrobial barrier function, and prevent the negative effects of topical GC.

Conflict of interest None declared.

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