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Co-treatment with retinyl retinoate and a PPARα agonist reduces retinoid dermatitis

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Abstract

Background Retinoids have been used for the treatment of skin disorders such as acne, psoriasis and photoaging. However, despite their beneficial effects, topical retinoids often cause severe local irritation called retinoid dermatitis. We previously developed a novel vitamin A derivative, retinyl retinoate, which induces less irritation and affords excellent tolerance. In this study, we examined whether co-treatment with topical peroxisome proliferator-activated receptor-α (PPARα) agonists (e.g. WY14643) reduce retinoid dermatitis in hairless mouse skin.

Methods The effect of concomitant treatment with a PPARα agonist on retinoid dermatitis in hairless mouse epidermis was evaluated by measuring transepidermal water loss, epidermal histology and cytokine expression.

Results Retinyl retinoate induced less severe retinoid dermatitis than retinoic acid. Topical application of a PPARα agonist improved the stratum corneum structure and function, reduced mRNA expression of interleukin (IL)-1α, tumor necrosis factor-α and IL-8, and inhibited ear edema induced by retinoic acid or retinyl retinoate.

Conclusions Our results indicate that PPARα agonists can potentially be used to improve retinoid dermatitis. We suggest that co-treatment with retinyl retinoate and a PPARα agonist may reduce or prevent detrimental alterations in retinoid-treated skin.

Introduction

Vitamin A and its derivatives, the retinoids, are involved in a wide range of biological processes, including cellular proliferation, differentiation and apoptosis.[1] Natural and synthetic retinoids are used to treat skin disorders such as acne, psoriasis, ichthyoses, hyperkeratotic disorders (keratodermas), cutaneous malignancies and photoaging.[2] However, retinoids cause a variety of adverse effects, including skin irritation. Patients using topical retinoid therapy often experience erythema, scaling, dryness, burning and pruritus. These symptoms characterize the retinoid-specific irritant contact dermatitis, commonly termed retinoid dermatitis.[3] This has led researchers to seek new retinoid derivatives that mimic the positive effects of retinoids without causing problematic side-effects. Damage to the skin barrier leads to enhanced transepidermal water loss (TEWL) and increased penetration of deeper

epidermal layers of living keratinocytes by further irritants. In a previous report, the side-effects of retinoic acid and instability of retinol were overcome by esterification with retinol and retinoic acid.[4,5] We previously synthesized a new hybrid retinoid, retinyl retinoate, via a condensation reaction between retinol and retinoic acid.[4] Retinyl retinoate overcomes several of the disadvantages of retinol and retinoic acid for application in cosmetics and over-the-counter drugs.[4,5] Retinyl retinoate caused less irritation than retinoic acid when applied topically to rodent skin[5] and, because of its excellent stability, mildness and higher efficacy in the skin, it represents a new synthetic retinoid that may have potential use as a cosmeceutical ingredient for the prevention and improvement of skin wrinkles or as a medicinal ingredient to treat aging skin.[4] In this study, we investigated whether agonists of peroxisome proliferator-activated receptors (PPARs) and liver X receptor (LXR) would alleviate retinoid dermatitis because such agents may offer interesting opportunities to improve the treatment of skin diseases, including disorders of inflammation, cell hyperproliferation and aberrant differentiation.[6,7] Peroxisome proliferator-activated receptors are ligand-activated transcription factors belonging to the nuclear receptor superfamily and include PPARα, PPARβ/δ and PPARγ.[8] The activators of PPARα, PPARα/β and LXRα/β include lipid synthetic intermediates or metabolites, such as certain free fatty acids, leukotrienes, prostanoids and oxygenated sterols.[9] Topical PPARα, -β/δ, and -γ agonists have been found to increase lipid synthesis in mouse epidermis *in vivo*.[6] In addition, PPARα, -β/δ and -γ ligands show an anti-inflammatory effect and decrease the inflammation induced by 12-O-tetradecanoylphorbol-13-acetate treatment, a model of irritant contact dermatitis.[10] PPARα agonists also reverse barrier abnormalities, epidermal hyperplasia and cutaneous inflammation.[7,11,12] Therefore, they show promise as candidate therapeutic agents in dermatology. The aims of this study were to compare the efficacy of retinyl retinoate and retinoic acid in reducing retinoid dermatitis and to investigate the effect of co-administration of a PPARα agonist on epidermal homeostasis.

Materials and methods

Animals and topical application

Female hairless mice (Skh1/Hr), aged approximately 6–8 weeks, were fed a standard mouse diet and water *ad libitum*. Groups of animals ($n = 4$) were treated topically with one of the following agents once daily for 3–5 d depending on the degree of irritation: 0.05% retinyl retinoate (Enprani Co. Ltd, Incheon, South Korea); 0.05% all-*trans* retinoic acid (Sigma Chemical Co., St Louis, MO, USA), or 0.05% WY14643 (Sigma Chemical Co.). Mouse dorsal skin was co-treated with 0.05% all-*trans* retinoic acid, or 0.05% retinyl retinoate and 0.05% WY14643, or vehicle only (propylene glycol : ethanol, 7 : 3 v/v) as the control. After application of retinoids and PPARα agonists once daily from baseline for 5 d, TEWL was measured using a Tewameter TM210 (Courage & Khazaka Electronic GmbH, Cologne, Germany).

Reverse transcription-polymerase chain reaction

To separate the epidermis from the underlying dermis, dorsal skins were floated on 10 mM ethylenediaminetetraacetic acid at 37 °C for 30 min, after which the epidermis could be pulled away from the underlying tissue with forceps (number of biopsies = 4). RNA was isolated from single nuclei using RNAzol (Invitrogen, Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Tissue samples were homogenized in 1 ml RNAzol reagent per 50–100 mg tissue using a tissue homogenizer for purification of total RNA. The mRNA levels were analyzed by reverse transcription (RT) and subsequent polymerase chain reaction (PCR) using an RNA PCR Kit (AMV) Version 2.1 (Takara Bio Inc., Kyoto, Japan) according to the manufacturer's instructions with the following modifications: the mixture was incubated for 30 min at 10 °C to extend the primers for effective annealing followed by RT for 1 h at 42 °C. The following primer pairs were used: mouse interleukin-1α (IL-1α), 5′-CTCTAGAGCACCATGCTACAGAC-3′ and 5′-TGGAATCCAG-GGGAAACACTG-3′ (310 bp); mouse tumor necrosis factor-α (TNF-α), 5′-GGCAGGTCTACTTTAGA-GTCATTG-3′ and 5′-ACATTCGAGGCTCCAGTGAATT-CGG-3′ (277 bp); mouse IL-8, 5′-CGCTCGCTTCTCTGTGCA-3′ and 5′-ATTTTCT-GAACCAAGGGAGCT-3′ (242 bp), and β-actin, 5′-TGGAATCCTGTGGCATCCATGAAAC-3′ and 5′-TAAAACGCAGCTCAGTAA-CAGTCCG-3′ (349 bp). The reaction was stopped by heating for 5 min at 95 °C. Polymerase chain reaction was performed with Ex *Taq* (Takara Bio Inc.) in Ex *Taq* buffer (Takara Bio Inc.) containing deoxynucleotide triphosphates. Amplification was carried out in a DNA Engine DYAD PCR machine (MJ Research, Inc., Waltham, MA, USA). Reaction products were electrophoresed in 2% agarose gels and visualized with ethidium bromide. The mRNA levels were quantified using TINA Version 2.10 (Raytest GmbH, Straubenhardt, Germany) and normalized to β-actin.

Immunohistochemical staining

Tissue was fixed in 4% formaldehyde, embedded in paraffin and processed for histological analysis. Sections were cut to 4 μm, mounted onto slides, deparaffinized and rehydrated in a graded ethanol series. Endogenous peroxidase was inactivated with 3% hydrogen peroxide in Tris-buffered saline. The sections were incubated in Protein Block Serum-Free Ready-To-Use (DakoCytomation Inc., Carpinteria, CA, USA) and incubated with anti-proliferating cell nuclear antigen (anti-PCNA) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), anti-loricrin (Covance Research Products, Inc., Richmond, CA, USA) or anti-involucrin (Covance Research Products, Inc.) antibodies in

Dako diluent at 4 °C overnight. The slides were incubated with streptoavidin-peroxidase (DakoCytomation, Inc.) or horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) at room temperature for 30 min, washed again with the buffer and incubated for 5 min in diaminobenzidine solution (DakoCytomation, Inc.). Finally, the sections were washed in distilled water and subjected to Mayer's hematoxylin staining.

Mouse ear edema measurement

Ears of animals were topically treated with one of the following agents once daily for 3 d: 0.05% all-*trans* retinoic acid; 0.01%, 0.05%, 0.1% or 1% WY14643, or vehicle only (ethanol) for the control. Retinoate was excluded because it does not cause much skin irritation. Edema was measured as the increase in ear thickness and ear weight caused by inflammatory change. Ear thickness was measured with a dial thickness gauge. To evaluate ear weight, animals were anesthetized, ear punch biopsies were collected using an 8-mm diameter punch, and biopsies were individually weighed on a Mettler-Toledo (AB-204-S) balance. Sections of mouse ears were subjected to hematoxylin and eosin staining.

Statistics

All data are presented as the mean ± standard error of the mean (SEM). Statistical differences between two groups were determined by a two-tailed Student's *t*-test. A *P*-value of <0.05 was considered significant.

Results

PPARα agonist inhibited the retinoid-induced increase in TEWL

The effects of PPARα and LXR agonists on TEWL induced by retinyl retinoate or retinoic acid were investigated by the co-application of these agents to hairless mouse skin. Palmitic acid and WY14643 were selected as PPARα agonists because they are widely used in the manufacture of cosmetics and drugs. TO901317 and 22(R)-hydroxycholesterol were used as LXR agonists. The increase in TEWL caused by retinoic acid or retinyl retinoate was reduced by both PPARα and LXR agonists (Fig. 1a,b). In particular, WY14643 (PPARα agonist) and 22(r)-hydroxycholesterol (LXR agonist) were more effective at blunting the TEWL increase caused by the

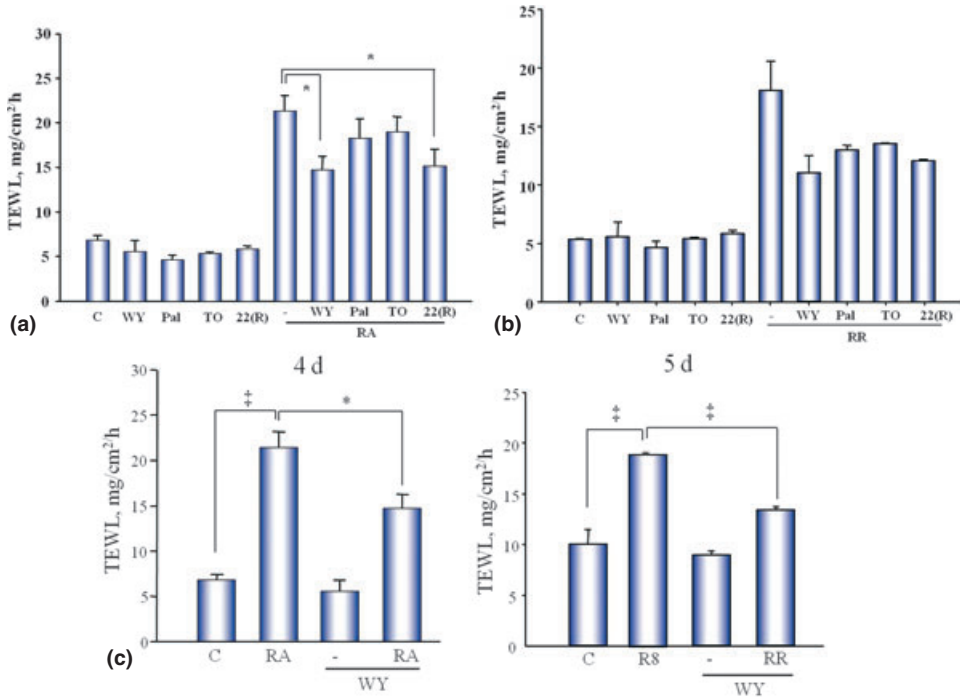

Figure 1 Effects of peroxisome proliferator-activated receptor-α (PPARα) and liver X receptor (LXR) agonists on increase of transepidermal water loss (TEWL) induced by retinoic acid (RA) and retinyl retinoate (RR): (a) PPARα agonist and LXR agonist, or RA alone, or co-treatment each applied at a dose of 0.05% once daily for 3 d; (b) RR applied for 6 d in mouse dorsal skin, and (c) PPARα agonist WY14643 applied alone or in co-treatment at a dose 0.05% once daily for 4 d (RA) or 5 d (RR). C, vehicle; WY, WY14643 (PPARα agonist); Pal, palmitic acid (PPARα agonist); TO, TO901317 (LXR agonist); 22(R), 22(R)-hydroxycholesterol (LXR agonist); $^{*}P < 0.05$; $^{\dagger}P < 0.001$

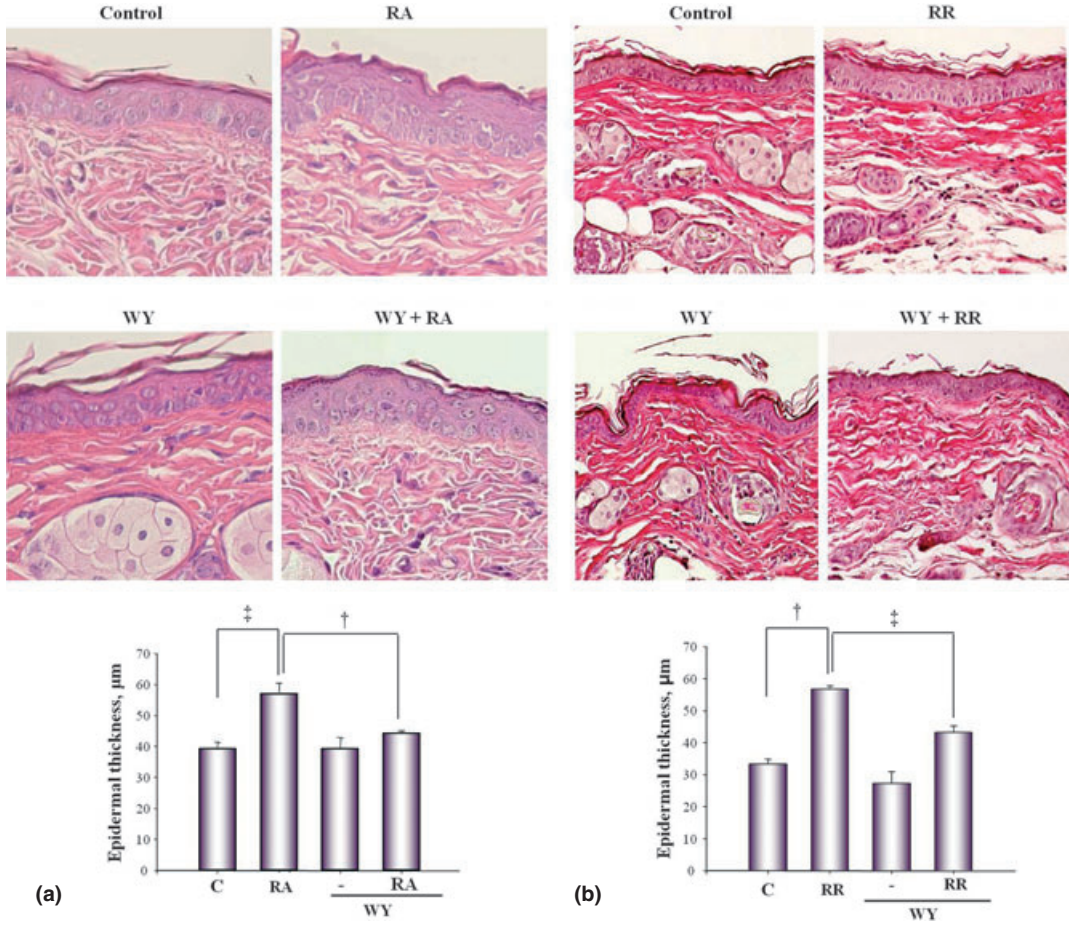

Figure 2 Effects of peroxisome proliferator-activated receptor-α (PPARα) agonist on epidermal thickness induced by retinoids. Retinoids, PPARα agonist WY14643 and their co-treatment were all applied at a dose of 0.05% once daily for (a) 4 d (retinoic acid) or (b) 5 d (retinyl retinoate). Sections of mouse skin were stained with hematoxylin and eosin (original magnification ×400). The thickness of the epidermal layer was measured in micrometers under microscopy at ×400 magnification. C, vehicle; WY, WY14643 (PPARα agonist); RA, retinoic acid; RR, retinyl retinoate; $^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.001$

retinoids than palmitic acid and TO901317, respectively (Fig. 1a). Therefore, in subsequent experiments, WY14643 was used as a PPARα agonist. Retinyl retinoate induced less skin barrier disruption than retinoic acid, as previously reported.[5] The optimal application times required for retinoic acid and retinyl retinoate to induce similar levels of irritation were 4 and 5 d, respectively (Fig. 1c). The increase in TEWL caused by both retinoids was reduced by approximately 36% by WY14643 ($P < 0.001$) (Fig. 1c).

PPARα agonist reduced retinoid-induced epidermal thickness, proliferation and differentiation

As shown in Fig. 2, retinoid treatment increased epidermal thickness compared with that in untreated animals. By contrast, the topical PPARα agonist WY14643 significantly reduced epidermal thickness (Fig. 2a). The increase in epidermal thickness caused by both retinoids was inhibited by co-treatment with WY14643 (Fig. 2a,b), as evidenced by the fact that the epidermis was thicker in the retinoid-treated group than in the group treated with both PPARα agonist and retinoid ($P < 0.05$). Cells treated with retinoic acid were highly proliferative and grew as a multilayer, with the expansion of proliferative keratinocytes as monitored by nuclear staining of PCNA, whereas in normal untreated hairless mouse skin, PCNA-positive cells were confined to the basal layer. The proliferation of epidermis cells caused by the 5-d application of retinyl retinoate was lower than that caused by the 4-d retinoic acid application (Fig. 3a,b) and lower epidermal hyperplasia was seen in retinyl retinoate-treated animals. Proliferation was higher in the epidermis of animals treated with either retinoid alone compared with that induced by retinoid and WY14643 co-treatment (Fig. 3a). To charac-

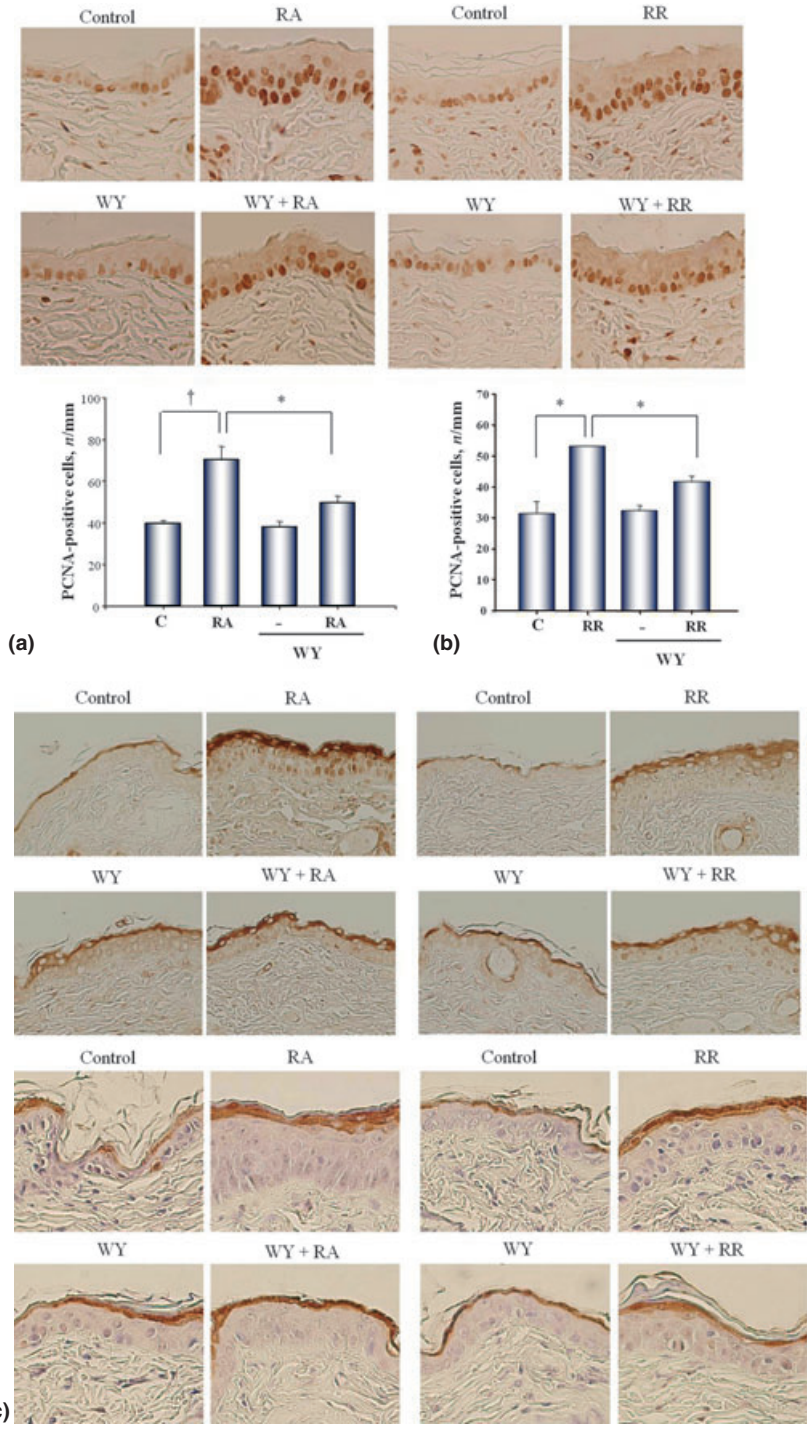

Figure 3 Effects of peroxisome proliferator-activated receptor-α (PPARα) agonist on retinoid-induced epidermal proliferation and differentiation: (a) retinoic acid (RA) and (b) retinyl retinoate (RR). Retinoids, PPARα agonist WY14643 or their co-treatment were all applied at a dose of 0.05% once daily for 4 d (RA) or 5 d (RR). Sections of mouse skin were stained with anti-proliferating cell nuclear antigen (anti-PCNA) antibody. (Original magnification ×400.) (c) Involucrin and (d) loricrin expression as differentiation markers were estimated by immunohistochemistry (×200). C, vehicle; WY, WY14643 (PPARα agonist); $^{*}P < 0.05$; $^{\dagger}P < 0.01$

terize the effect of PPARα agonists and retinoids on epidermal differentiation, we investigated expression of involucrin and loricrin, two markers of epidermal differentiation, by immunohistochemical analyses. Topical application of retinoids enhanced expression of involucrin and loricrin in the epidermis, whereas the PPARα agonist reduced it slightly (Fig. 3c,d). The induction of involucrin and loricrin by retinyl retinoate or retinoic acid was inhibited by WY14643.

PPARα agonist reduced retinoid-induced IL-1α, TNF-α and IL-8 mRNA expression

We also evaluated mRNA levels of cytokine mediators in retinoid-induced inflammation. Secretion of IL-1α, TNF-

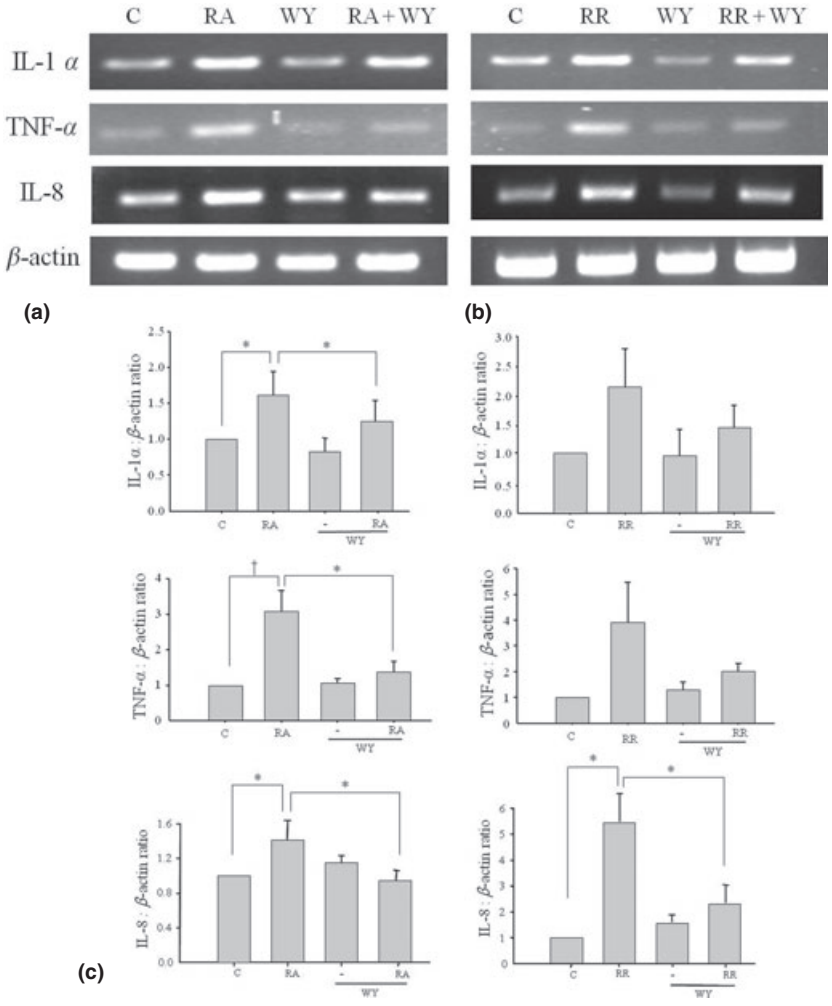

Figure 4 Effects of peroxisome proliferator-activated receptor-α (PPARα) agonist on retinoid-induced interleukin-1α (IL-1α), tumor necrosis factor-α (TNF-α) and IL-8 mRNA expression in hairless mouse epidermis. Retinoic acid, retinyl retinoate, WY14643 and co-treatment were applied at a dose of 0.05% once daily for 4 or 5 d. The mRNA expression of IL-1α, TNF-α and IL-8 was determined by reverse transcription polymerase chain reaction and quantified by TINA Version 2.10. Data were normalized with β-actin mRNA. C, vehicle; WY, WY14643 (PPARα agonist); RA, retinoic acid; RR, retinyl retinoate; $^{*}P < 0.05$; $^{\dagger}P < 0.01$

α, IL-6 and IL-8 has been demonstrated in response to irritants attached to the skin and these cytokines are thought to be important mediators in retinoid dermatitis.[13] Induction of these cytokines is a good indicator of retinoid-induced irritation. The mRNA levels of IL-1α, TNF-α and IL-8 were significantly increased by a 4-d application of retinoic acid compared with vehicle ($P < 0.05$), whereas 5-d retinyl retinoate treatment resulted in a non-significant increase in cytokine expression (Fig. 4a,b). The increase in IL-1α expression caused by retinoic acid or retinyl retinoate was inhibited by co-treatment with the PPARα agonist WY14643 (Fig. 4).

PPARα agonist reduced retinoic acid-induced ear edema

We next investigated inflammation in hematoxylin and eosin-stained sections of mouse ears treated with topical retinoic acid. The thickness and weight of ears, evidence of edema and epidermal hyperplasia, were significantly increased by treatment with retinoic acid. Co-treatment with PPARα agonist WY14643 reduced these responses in a dose-dependent manner (Fig. 5a). In addition, substantial inflammatory cell infiltration of the dermis with accompanying connective tissue disruption reflected the production of epidermal cytokines such as IL-1α (Fig. 4c), either as a result of direct retinoid-stimulated keratinocyte proliferation or as a consequence of the disruption of the epidermal barrier that accompanies retinoid stimulation. Co-treatment with the PPARα agonist alleviated the retinoid-induced inflammation (Fig. 5b).

Discussion

Retinoid dermatitis is a complex disease, the pathophysiology of which is poorly understood. It has been suggested that the irritation is related to epidermal hyperplasia characterized by epidermal hyperkeratosis and a thickened granular layer that results from the induction of cell proliferation in the epidermal basal layer within 24 h of retinoid application, as measured by DNA synthesis.[14] The increase in epidermal thickness and the increased

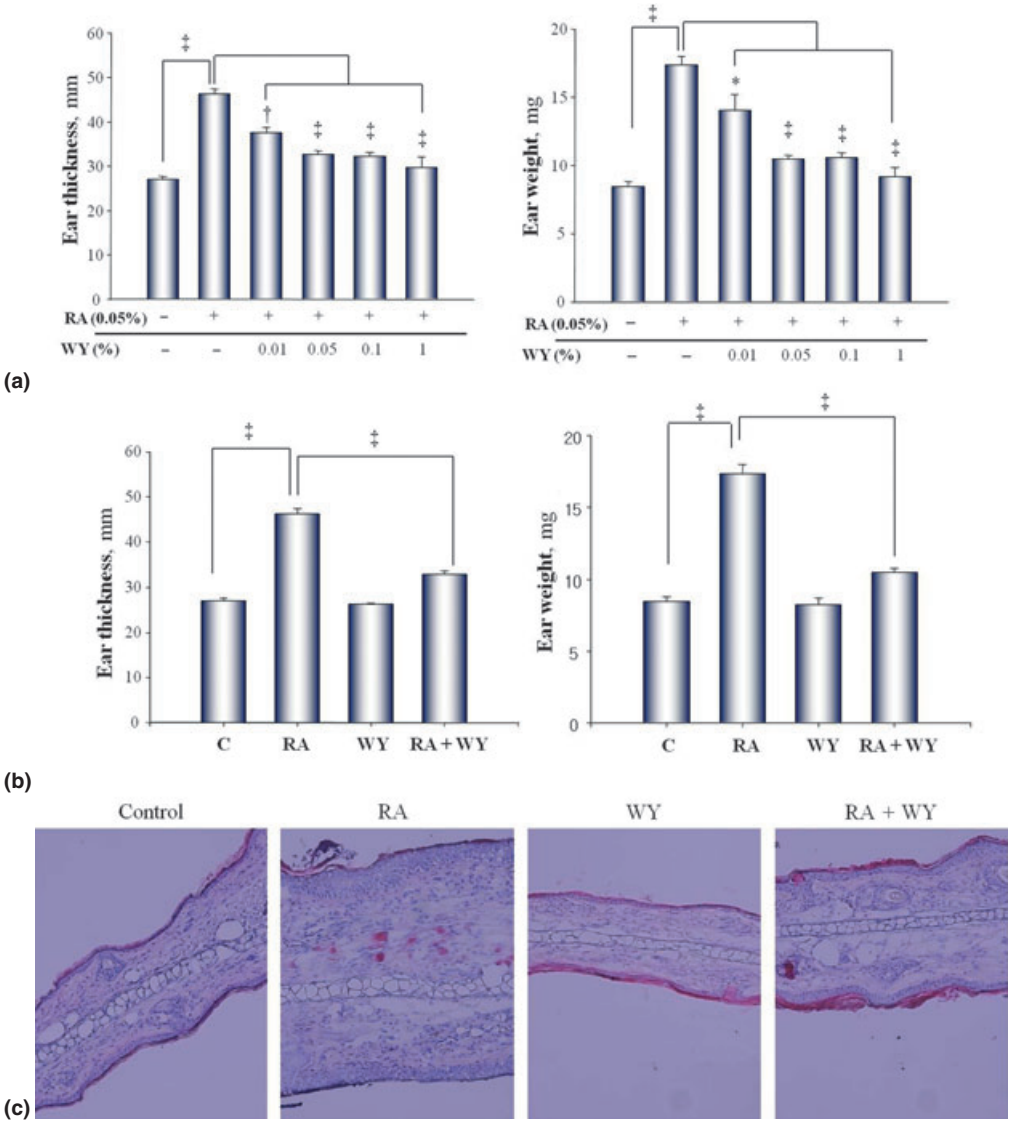

Figure 5 Effect of peroxisome proliferator-activated receptor-α (PPARα) agonist on retinoic acid (RA)-induced ear edema. (a) Edema was expressed by the increase in ear thickness and weight after once daily application of 0.05% RA and WY14643 serial doses for 3 d. (b) Edema was measured after co-application of 0.05% RA and 0.05% WY14643 for 3 d. (c) Sections shown are representative of observations in each group. (Hematoxylin and eosin stain; original magnification ×200.) C, vehicle; WY, WY14643 (PPARα agonist); $^{*}P < 0.05$; $^{\dagger}P < 0.01$; $^{\ddagger}P < 0.001$

number of epidermal cell layers induced by retinoids indicate that these agents can potentially be used to stimulate cellular renewal. In addition to inducing hyperplasia, retinoid treatment alters the pattern of differentiation in the epidermis and reduces barrier function, which disrupts the integrity of the stratum corneum.[12] The reduction of retinoid-induced expression of involucrin and loricrin by the PPARα agonist indicates that PPARα agonists may play a role in the differentiation of keratinocytes. Erythema reflects the production of epidermal cytokines, either as a direct result of retinoid-stimulated keratinocyte proliferation or in consequence to the disruption of the epidermal barrier that accompanies retinoid stimulation.[15] Although it has been reported that rapidly proliferating keratinocytes produce large amounts of proinflammatory cytokines,[16] loss of the skin barrier function, rather than hyperproliferation, may be directly responsible for promoting proinflammatory events. In this study, we showed that retinyl retinoate is similar to retinoic acid in its ability to induce epidermal hyperplasia in hairless mouse epidermis. However, retinyl retinoate caused less severe irritation than other retinoids when applied to the dorsal

skin of hairless mice, in agreement with previous data for cell toxicity, human primary irritation and hairless mouse skin irritation tests.[4,5] Consistent with these findings, retinoic acid induced higher levels of transcription of the proinflammatory cytokines than retinyl retinoate. Furthermore, the induction of proinflammatory cytokines by retinyl retinoate was reduced by the PPARα agonist WY14643. In a previous study, application of retinoic acid to hairless mouse skin significantly increased TEWL at 3 d ($P < 0.001$),[5] whereas application of PPARα, -β/δ and -γ agonists or LXR agonists alone did not affect TEWL. However, the PPARα agonist WY14643 and the LXR agonist TO901317 significantly reduced the increase in TEWL caused by retinoic acid (data not shown). In an animal model of epidermal hyperplasia, epidermal morphology was restored to normal by treatment with oxysterol (an LXR agonist) through both inhibition of proliferation and stimulation of differentiation,[17] and the cutaneous inflammation associated with irritant and allergic contact dermatitis was markedly reduced by topical treatment with PPARα agonists. Our study also demonstrates the anti-inflammatory effects of PPARα agonists. The proliferation of epidermis was slightly greater in the group treated with the retinoid than in the group co-treated with the PPARα agonist and retinoid. Our data indicate that the PPARα agonist inhibits proliferation and stimulates differentiation for epidermis homeostasis. Treatment with retinoids induces expression of IL-1α, TNF-α and IL-8. Interleukin-1α induces a number of events in vascular endothelial cells that ultimately result in increased vascular permeability and leukocyte influx,[18] whereas IL-8 plays a central role in retinoid-induced skin irritation.[19] It is widely recognized that TNF-α and IL-1α, which are secreted in response to injury, are key mediators of the cutaneous inflammatory response.[20] Retinoic acid induces IL-8 production independently of both nuclear factor-κB (NF-κB) and p38 mitogen-activated protein kinase in normal human keratinocytes.[19] It is likely that multiple mechanisms are involved in the down-regulation of the inflammatory response and that the induction of the PPARα pathway is sufficient to restore homeostasis. In this study, we demonstrated that topical treatment with PPARα agonists inhibits the expression of cytokines and mouse ear edema caused by retinoids. Stimulation by PPARα was also shown to reduce the cytokine-induced activation of a number of proinflammatory genes and thus the anti-inflammatory effects of PPARα activation may result from the inhibition of both TNF-α and IL-1α. The transcription of cytokines such as TNF-α, IL-1α and IL-8, and many of the effectors of cytokine action, such as vascular cell adhesion molecule-1 and cyclo-oxygenase-2, are regulated by NF-κB. PPARα may interfere with NF-κB by direct protein–protein interaction, or may antagonize activation by inducing inhibitor of NF-κB (IκB-α), thus accelerating NF-κB deactivation.[21] The role of PPARα in the inhibition of the inflammatory response was also linked to the degradation of immune modulators such as leukotriene B4 and the induction of anti-oxidant enzyme catalase, which reduces oxidative stress.[22]

To summarize, this study shows that topical retinoic acid induces an increase in IL-1α, IL-8 and TNF-α expression, which suggests that these cytokines mediate inflammatory signaling and play a pivotal role in retinoid-induced acute irritant contact dermatitis. The study also shows that the PPARα agonist WY14643 inhibits retinoid-induced inflammation in an ear edema mouse model. Coupled with the previously described pro-differentiation and anti-proliferative effects of PPARα agonists, these data indicate the potential use of such agents in the treatment of a variety of cutaneous disorders. Our findings further demonstrate that retinyl retinoate causes less damage to the skin than retinoic acid and that co-treatment with retinyl retinoate and a PPARα agonist is more effective than treatment with retinoid alone in topical retinoid therapy.

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