Reduced Retinoid Signaling in the Skin after Systemic Retinoid-X Receptor Ligand Treatment in Mice with Potential Relevance for Skin Disorders

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Key Words

Retinoid-X receptor ligand • Retinoids • Skin disorder

Abstract

Retinoid-X receptor (RXR)- and retinoic acid receptor (RAR)-mediated signaling is induced by retinoic acids (RA), which are involved in the regulation of skin permeability, differentiation and immune response. Dysregulation of retinoid signaling is present in various skin disorders. Topically and systemically administered synthetic RAR or RXR agonists might influence retinoid-mediated signaling in the skin of RARE reporter animals and gene expression analysis for retinoid, skin homeostasis and skin inflammation marker genes and local retinoid concentrations. Mice were treated orally and topically with synthetic ligands and bioimaging, QRT-PCR and retinoid analysis were performed. Topical application of the synthetic RAR ligand AM580 significantly enhanced retinoid signaling in skin while topical application of the RXR ligand LG268 did not influence retinoic acid receptor response elements (RARE)-mediated signaling. Systemic treatments with LG268 decreased the expression of genes involved in skin homeostasis, RA synthesis and skin RA concentrations, while it increased various markers for skin

inflammation and RA degradation, which corresponds to decreased skin RARE signaling. We conclude from these observations that increased systemic concentrations of an RXR ligand may be one reason for reduced retinoid signaling, reduced all-*trans* RA levels in the skin, reduced epidermal homeostasis and increased skin inflammation marker expression with potential relevance for various skin disorders, like atopic dermatitis.

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Introduction

Retinoids are essential for normal physiological regulation of many organs, tissues and cell types. The immune system and the skin are classical targets for retinoid function [1, 2]. Numerous features or processes are regulated by retinoids in cells related to these targets; e.g. epidermal permeability (based on epidermal hyper-proliferation), epidermal differentiation [3], sebum secretion [4], Th1/Th2 balance [5], apoptosis [6] and bacterial colonization [7]. These processes are also severely dysregulated in selected skin disorders and especially atopic dermatitis (AD) and are also co-regulated by endogenous retinoids.

In addition, various skin and immune diseases respond to systemic and oral pharmacological treatments with retinoids [1, 8].

In a previous study, we observed strongly reduced retinoid signaling and retinoid concentrations in affected as well as nonaffected skin of AD patients as compared to healthy individuals [9]. It is possible that this reduced retinoid signaling in the skin might be a key step for the development and/or chronification of AD. Various other studies have reported that the retinoid-X receptor (RXR) seems to play an important role in the initiation of allergic sensitization in mice [10, 11]. In epidermal-specific RXR knockout mice (RXR $\alpha\beta^{ep-/-}$), an AD-like chronic dermatitis could be observed, which is very similar to that found in AD patients [12]. Systemic application of an RXR ligand has the potential to enhance systemic Th2 development [5]. Furthermore, increased expression of Th2 cytokines is a key feature of various atopic diseases including AD [11, 13, 14].

In this study we have used transgenic reporter mice containing retinoic acid receptor response elements (RAREs) coupled to luciferase and quantitative reverse transcription-polymerase chain reaction (QRT-PCR) for expression analysis of retinoid target genes in orally gavaged as well as topically treated animals to study retinoid signaling related to skin disorders like atopic dermatitis.

The aim of this study was to elucidate how the administration of synthetic selective activators of retinoic acid receptors (RAR) or RXR as well as vitamin A supplementation might influence the retinoid signaling in the skin with respect to skin inflammation and skin homeostasis relevant for skin disorders like atopic dermatitis.

Materials and Methods

Mice

Six-week-old female C57BL/6 mice were purchased from Charles River, Hungary and housed at the Laboratory Animal Core Facility of the University of Debrecen. Mice were kept at 22°C RT and 12 h light/dark cycle. During 2 weeks of acclimatization mice were maintained on a standard chow diet with 5% fat content (VRF1, Altromin, Germany) and had water ad libitum. All animal experiments were performed in Debrecen, Hungary, and approved by the Committee of Animal Research of the University of Debrecen.

Oral and Topical Application of Retinoids

Oral gavage with specific synthetic nuclear hormone receptor ligands, dissolved in 25% Cremophor EL (Sigma-Aldrich, Hungary)/water (v/v) was performed at 5 ml/kg body weight. The vehicle (Cremophor EL) was applied as negative control also at 5 ml/kg body weight. Mice were treated with the RAR ligand AM580

(Biotrend Chem. GmbH, Cologne, Germany) at a concentration of 10 mg/kg body weight or the RXR ligand LG268 (Ligand Pharmaceuticals, San Diego, Calif., USA) at a concentration of 30 mg/kg body weight.

The topical application of the specific synthetic nuclear hormone receptor ligands was performed on a freshly shaved 1 × 1 cm surface on the dorsal area of the mouse skin using each time 25 μ l isopropanol solution with a concentration of 40 nmol/25 μ l AM580 or LG268 100 nmol/25 μ l. Mice were bioimaged and consecutively sacrificed 18 hours after the application. Skin samples were immediately frozen in liquid nitrogen after dissection and kept at -80°C until RNA extraction.

Bioimaging

RARE-Luc mice (n = 6) were obtained from Cgene, Oslo, Norway [15, 16] and were given an oral gavage with AM580 or LG268 once. The topical and oral application of the ligands was performed like previously mentioned. Experiments were performed 18 h after retinoid applications. Mice were anesthetized with 10 mg/ml pentobarbital, then injected with 120 mg/kg body weight luciferin and placed in a light tight chamber. Bioimaging was conducted using an Andor-IXON CCD camera and analysis was performed by Andor-IQ software. After the imaging the integrated intensity/area was calculated for skin and liver of each treated animal.

Supplementation with Low, Normal and High Vitamin A Diet

Female C57BL/6 mice (6–8 weeks old) were fed with a vitamin A-deficient diet for 10 weeks, followed by supplementation with low (0 RE/kg diet), normal (2,500 RE/kg diet), or high (324,000 RE/kg diet) vitamin A for 4 weeks. The chow consisted of wheat starch (Weizenstärke, Foodstar, Germany) provided by Kröner-Stärke (Ibbenbüren, Germany), saccharose (purchased from a local supermarket in Hungary), casein (Sigma-Aldrich, Hungary), cellulose vivapur (Pharma GmbH & Co KG, Germany), vitamin mix (Vitamin-Vormischung C1000, Altromin GmbH, Germany), mineral mix (Mineral-Spurenelemente-Vormischung C100, Altromin). The diet was prepared manually. Skin samples were immediately frozen in liquid nitrogen after dissection and kept at -80°C until RNA extraction.

High-Performance Liquid Chromatography Mass

Spectrometry – Mass Spectrometry (HPLC MS-MS) Analysis

Concentrations of all-*trans* retinoic acid (ATRA) were determined in mouse skin samples by our HPLC-MS-MS method [17]. Briefly, 100 mg of the skin biopsy (if samples were under 100 mg, water was added up to the used standard weight: 100 mg) was diluted with a threefold volume of isopropanol, the tissues were minced by scissors, vortexed for 10 s, put in an ultrasonic bath for 5 min, shaken for 6 min and centrifuged at 13,000 rpm in a Heraeus BIOFUGE Fresco at +4°C. After centrifugation, the supernatants were dried in an Eppendorf concentrator 5301 (Eppendorf, Germany) at 30°C. The dried extracts were resuspended with 60 μ l of methanol, vortexed, shaken, diluted with 40 μ l of 60 mM aqueous ammonium acetate solution and transferred into the autosampler and subsequently analyzed.

mRNA Expression Analysis

Total RNA was isolated from skin samples using Tri[®] reagent solution (Invitrogen, Hungary) according to the manufacturer's

Fig. 1. Bioluminescence imaging of RA-RE-Luc mice upon systemic or topical treatment with RAR (AM580, AM) or RXR (LD268, LG) activators (integrated intensity/area normalized to control, control is set as 1). ND = Not determined; CTRL = control. * $p < 0.05$.

instructions. For real-time quantitative PCR (QRT-PCR), total RNA was reverse transcribed into cDNA. QRT-PCR was carried out using predesigned MGB assays or FAM-TAMRA assays ordered from Applied Biosystems, on an ABI Prism 7900. Relative mRNA levels were calculated using the comparative threshold cycle (C_T) method and were normalized to the cyclophilin A mRNA level. For data analysis Sequence Detector Software (version 2.1) was utilized.

Statistics

The data are indicated as mean of triplicate measurements and standard error mean values per data point. One data point stands for 6 animals. Statistical analysis was performed using the program SPSS 16.0. $p < 0.05$ was considered significant.

Results

Systemic Treatment of RARE Reporter Animals with an RXR Ligand Results in Downregulation of RARE Activity in the Skin (fig. 1)

With bioluminescence imaging of RARE-Luc mice upon topical treatment with the RAR ligand AM580, we found a significant upregulation of RARE response in the skin, while topical treatment with LG268 resulted in no significant change in RARE-response. Systemic treatment of mice with AM580 did not cause any alteration of RARE response in the skin, while in the liver the RARE response was significantly upregulated. After oral administration of LG268, a significant decrease of RARE response was observed in the skin, while it increased in the liver.

Systemic Treatment with a Synthetic RXR Ligand Results in Reduced RARE Response Which Is Also Confirmed by Reduced RA Synthesis and Increased RA Degradation in Mouse Skin and Reduced Skin RA Concentrations (fig. 2)

To confirm the data achieved by bioluminescence imaging we performed the gene expression analysis of enzymes responsible for RA synthesis (Raldh 1, Raldh 2 and Raldh 3) and RA degradation (Cyp26a1, Cyp26b1) in the skin was additionally performed using QRT-PCR analysis (fig. 2a). Firstly, the expression of the major RA synthesis enzyme Raldh 1 was strongly decreased after systemic AM580 or LG268 treatments, while Raldh 2 and 3 were increased. Expression of RA-degrading enzymes Cyp26a1 was not significantly affected, while Cyp26b1 increased after AM580 or LG268 treatments. Expression of the intracellular RA-transporter Crabp2 was increased after AM580 or LG268 treatments (fig. 2b). Secondly, when summarizing the expression of RA-synthesizing and RA-degrading enzymes a decrease could be observed for RA synthesis after AM580 or LG268 treatment, while for RA degradation an increase could be observed only after LG268 treatment (fig. 3a). Thirdly, the RARE response and QRT PCR-based data about RA synthesis versus RA degradation were confirmed by the product for RA synthesis and substrate for RA degradation and additional all-*trans* retinoic acid concentrations by HPLC-MS-MS measurement in the skin (fig. 2c). After AM580 or LG268 treatment, a decrease of the RA concentrations in the skin was observed, while retinol levels remained almost stable. To better explain the RA synthesis versus

Fig. 2. **a** mRNA expression of RA-synthesizing enzymes Raldh 1, Raldh 2, Raldh 3 and RA-degrading enzymes Cyp26a1 and Cyp26b1 in mouse skin after oral gavage of ligands. **b** mRNA expression of Crabp2. **c** Concentration of ATRA and retinol in the skin of CTRL, AM- and LG-treated mice, respectively, product/substrate ratio of ATRA versus retinol. * $p < 0.05$.

Fig. 3. **a** Summarized mRNA expression level of Raldh 1, Raldh 2, Raldh 3 and Cyp26a1 and Cyp26b1 in mouse skin after single oral gavage of ligands. **b** Summarized table of influence of systemic or topical treatments with synthetic retinoids on retinoid synthesis, retinoid degradation, Crabp2 transport, RA application, skin ATRA levels and RARE response with relevance for positive and negative influence on retinoid signaling. CTRL = Vehicle treatment; AM = AM580; LG = LG268.

Fig. 4. mRNA expression of the RAR target genes Crabp1, Hbegf, Crabp2 and Tslp in mouse skin after supplementation diets with deficient (D), normal (N) and high (H) vitamin A concentrations. * $p < 0.05$ versus animals fed a normal vitamin A diet.

RA degradation ratio and the resulting RARE response, a product versus substrate ratio for ATRA versus retinol was calculated (fig. 2c). Based on this analysis, we found that this ratio was much lower after LG268 treatment compared with vehicle and AM580 treatments. Finally, when we summarized the expression data of RA synthesis enzymes, RA degradation enzymes, intercellular RA transporter Crabp2, treatment with AM580 and the skin ATRA concentration and the resulting skin RARE response (fig. 3b), we can explain why we observed a decrease in skin RARE-response after systemic LG268 treatment.

Regulation of Specific Skin Homeostasis and Skin Inflammation Marker Genes (table 1)

Various skin specific markers of AD involved in skin homeostasis and skin inflammation were also analyzed via QRT-PCR. We observed that the skin inflammation and homeostasis marker genes like thymic stromal lymphopoietin (Tslp), filaggrin (Flg), beta-glucocerebrosidase (Gba) and serine palmitoyltransferase (Spt1c2) were significantly decreased, while heparin-binding EGF-like growth factor (Hbegf) and involucrin (Ivl) were just slightly and nonsignificantly decreased.

The marker genes for skin inflammation and itch/scratch response such as leukotriene B4 receptor 2 (Blt2, Ltb4r2), thromboxane receptor (Tbxa2r) and thromboxane synthase (Tbxas1) showed significantly higher mRNA expression levels indicating the presence of a skin-based inflammation in the skin. Especially TXB2-,

Table 1. mRNA expression of genes involved in retinoid metabolism, skin homeostasis and skin inflammation in mouse skin after oral applications of LG268

Gene code	Gene name	Relative gene expression	Significance
Tslp	thymic stromal lymphopoietin	0.41 ± 0.19	0.05
Gba	beta-glucocerebrosidase	0.68 ± 0.16	0.001
Spt1c2	serine palmitoyltransferase	0.26 ± 0.13	0.006
Flg	filaggrin	0.23 ± 0.37	0.03
Hbegf	heparin-binding EGF-like growth factor	0.80 ± 1.30	0.85
Ivl	involucrin	0.87 ± 0.07	0.64
Alox5	arachidonate 5-lipoxygenase	8.98 ± 0.12	0.005
Ltb4r2	leukotriene B4 receptor 2	1.47 ± 0.08	0.03
Tbxa2r	thromboxane receptor	278 ± 0.10	0.002
Tbxas1	thromboxane synthase	15.8 ± 0.14	0.06

Control is set as 1.

Alox5- and Blt2-mediated signaling is also associated with AD specific itch/scratch response [18–20].

High Dietary Vitamin A Supplementation Results in Upregulation of RAR Target Gene Expression in Mouse Skin

We determined the expression of the retinoid target genes Crbp1, Crabp2, Hbegf and Tslp in the skin of mice supplemented with a low or high vitamin A diet for

4 weeks. The gene expression of mRNA levels of these retinoid target genes were significantly increased in the skin of mice supplemented with a diet high in vitamin A in comparison to the skin from animals fed a vitamin A-deficient and normal vitamin A diet (fig. 4).

Discussion

Retinoid-mediated gene expression is important for the skin and the immune system as displayed in cultured keratinocytes and human skin [21, 22]. Dysregulation of retinoid metabolism is either the cause or the initiator of various diseases including allergic diseases [23, 24]. Reduced concentrations of ATRA and retinol as well as reduced retinoid signaling are present in affected as well as nonaffected skin of AD patients [9]. In our studies, we observed that systemic treatment of a synthetic RXR ligand leads to reduced retinoid signaling in mouse skin and therefore it might possibly be relevant for reduced retinoid concentrations and retinoid signaling in mouse skin. We suggest that an aberrant systemic retinoid status could be an initiator of dysregulated skin inflammation and skin homeostasis present in various skin disorders especially atopic dermatitis.

So far, two potential RXR ligands are known: (1) 9-*cis* retinoic acid [25], an ATRA isomer, which was never convincingly detected by competent analytical expert groups [17, 26–29] in the mammalian organism in sufficient concentrations to initiate gene expression, and (2) docosahexaenoic acid (DHA), a free fatty acid [30–32], which should obtain RXR-activating potential, as well as sufficient endogenously relevant concentrations. However, DHA's role as an endogenous RXR ligand has never been convincingly demonstrated. Thus, it is possible that other endogenous RXR ligands exist, and our group together with cooperation partners is searching for endogenous RXR ligands in order to determine whether their concentrations are dysregulated in the immune system, skin and serum of atopy patients as well as in animal models for allergic sensitization.

RXR is present endogenously in three isotypes (RXR α , RXR β and RXR γ) with a distinct temporal and spatial expression pattern within the mammalian organism [33, 34]. The RXRs can form heterodimers with various nuclear receptors like the peroxisome proliferator-activated receptors (PPARs), liver-X receptors (LXRs), RARs, vitamin D receptor (VDR) and the nuclear receptor subfamily 4, group A, member 1 and 2 receptors (NR4A1/Nur77 and NR4A2/Nurr1). Further, they are required for tran-

scriptional homodimer-mediated signaling [33, 34]. Skin specific RXR α $\beta^{\text{ep-/-}}$ epidermal knockout mice develop a skin similar to that of AD patients [12]. It can be concluded that RXR or its ligands are able to modify the initiation of allergic sensitization [5, 11, 35]. In our experiments using a synthetic RXR ligand we could clearly show that its systemic application results in a strong reduction of RAR-mediated signaling in skin and this could be possibly the cause for dysregulated skin inflammation and homeostasis present in AD skin phenotype [12, 36].

In order to explain this reduced RARE-mediated signaling after systemic pharmacological RXR ligand application, we assessed expression patterns of the key enzymes involved in retinoid metabolism; the three RA-synthesizing enzymes retinal dehydrogenase (Raldh 1, 2 and 3) and the two major RA-degrading enzymes Cyp26a1 and Cyp26b1 (fig. 3a). We observed that systemic application of an RXR ligand results in a strong induction of RA-degrading enzymes compared to reduced RA-synthetic enzymes. As a result, we can conclude that this dysregulated induction of RA synthesis versus RA degradation enzymes may be the key to explain reduced RARE response, resulting in a reduction of retinoid-mediated signaling, reduced ATRA concentrations in the skin, reduced skin homeostasis marker gene and increased skin inflammation marker gene expression.

What may be the relevance of this phenomenon in respect to skin disorders with a special focus on AD? In general, retinoid dysregulation in affected as well as non-affected AD skin is one main feature of this disease [9]. In our study, thromboxane synthase and thromboxane receptor as well as 5-lipoxygenase and leukotriene B4 receptor [19, 20] increased after oral RXR ligand treatment, which concludes that itch and scratch responses, partly responsible for the AD phenotype, are also co-initiated via increased systemic RXR-mediated signaling [37]. In addition, the expression of other genes with relevance to the epidermal barrier like filaggrin, serine palmitoyl-transferase and beta-glucocerebrosidase are also strongly downregulated. Especially filaggrin has an important relevance for the epidermal barrier and mutations in its genes of are highly associated with increased AD risk [38]. That altered systemic RXR-mediated signaling may be an important factor for the reduced expression and thereby an important factor for AD and other skin diseases might be a possible explanation. The question is whether this dysregulation is a symptom or a cause of the skin AD phenotype. It has been shown previously that RXR signaling is involved in the immune phenotype of

atopy [5, 11, 12, 39] and here we tried to explore whether RXR-mediated signaling is also involved in the skin phenotype of AD. Using our experimental setup, we clearly determined a negative effect on mouse skin without prior systemic or topic sensitization, just by systemic RXR ligand application. The exact regulation of RXR signaling is not known and we attempted to determine the influence of vitamin A (retinol/retinyl esters) known as nutritional precursors of retinoids which can activate RXR-mediated, but also RAR-mediated signaling. A high vitamin A supplementation diet leads to increased expression of RAR target genes which is partly in contrast to our observed effects of reduced RAR-mediated signaling induced by systemic treatment with a selective RXR ligand.

The implication for humans might be that these RXR-mediated effects, via endogenous retinoids originating from food or various environmental derivatives like organotin compounds or plasticizers, may interfere with RXR signaling and be a trigger for the increased prevalence of atopy [40].

In summary, systemic pharmacological exposure to an RXR ligand induces reduction of retinoid signaling and target gene expression in the skin. As reduced retinoid signaling is present in skin disorders like AD, we postulate that increased systemic RXR ligand concentration in the organism could be one major reason for dysregulated skin inflammation/skin homeostasis which is an important initial step for the onset of allergic sensitization.

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Disclosure Statement

The authors declare no conflict of interest.

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