Decreased retinoid concentration and retinoid signaling pathways in human atopic dermatitis

Johanna Mihály[1], Anat Gamlieli[1], Margitta Worm[3], Ralph Rühl[1,2]

[1]Laboratory of Nutritional Bioactivation and Bioanalysis, Department of Biochemistry and Molecular Biology, University of Debrecen;
[2]Apoptosis and Genomics Research Group of the Hungarian Academy of Science, Debrecen, Hungary,
[3]Allergy-Center-Charité, Department of Dermatology and Allergology, Charité – Universitätsmedizin Berlin, Germany

Wordcount: 3959
Number of display items: 5

Corresponding author:

Dr. rer. nat. habil. Ralph Rühl
Department of Biochemistry
and Molecular Biology
Medical and Health Science Center
University of Debrecen
Nagyerdei krt. 98
H-4012 Debrecen
Tel: +36 30 2330 501
Fax: +36 52 314 989
E-mail: rruehl@dote.hu

Prof. Dr. med. Margitta Worm
Department of Dermatology
and Allergology
Charité-Universitätsmedizin Berlin

Charitéplatz 1
D-10117 Berlin
+ 49 30 450 518 105
+ 49 30 518 919
margitta.worm@charite-de

Abstract

Atopic dermatitis (AD) is one of the most common skin diseases. Various features present in AD like inflammation, reduced apoptosis, altered epidermal differentiation and hyperproliferation as well as permeability dysfunction are also regulated by retinoids.

The aim of our study is to identify the retinoid signaling pathways and retinoid concentration profiles in AD skin.

Human skin biopsies were obtained from healthy volunteers (HS) (n=6) and AD patients (n=6), with both affected (AS) and non-affected (NAS) skin. The gene expression of retinoid receptors, retinoid-binding proteins and retinoid-metabolizing enzymes was investigated by QRT-PCR. Retinoid concentrations in serum and skin were measured via high performance liquid chromatography mass spectrometry – mass spectrometry.

Our results show that the target gene expression of retinoid receptor regulated pathways is significantly decreased in AS and NAS of AD patients. CYP26A1, transglutaminase 2 and retinoic acid receptor responder 1 decreased in NAS and AS in comparison to HS. The main retinoic acid synthesizing enzyme, retinal-dehydrogenase 1, was significantly lower expressed in NAS (0,1%) and AS (1%) in AD patients. Analysis of retinoid concentration in serum and skin showed comparable all-*trans* retinoic acid (ATRA) and retinol (ROL) concentrations from AD and healthy serum, but strongly reduced ATRA and ROL concentrations in affected and non-affected skin in comparison to healthy skin.

Our data indicate that retinoid transport, synthesis, concentrations and signaling are strongly decreased in the affected but also in non-affected skin of atopic dermatitis patients suggesting a general intrinsic influence on skin retinoid signaling pathway in AD patients.

Keywords: retinoids, retinol, vitamin A, atopic dermatitis, gene expression

Introduction

Vitamin A and its derivatives, the retinoids, are important for skin physiology (1). Retinoids regulate various effects in differentiation, proliferation, apoptosis, immune regulation, barrier properties (2-3) and sensorial functions (4) in numerous skin cell types. Several skin diseases like psoriasis (5), ichthyosis (6), skin cancers (7), acne (8) and various other dermatoses are related to alterations in retinoid metabolism / signaling. Retinoid-based treatments have been shown to be beneficial for various therapeutic approaches of these skin diseases (9). Topical as well as systemic treatments with retinoids like, retinoic acid and synthetic retinoic acid analogues or derivatives which modify retinoid metabolism are used already in therapy (1, 10-12).

Retinoids mainly mediate their activity via nuclear hormone receptors, the retinoic acid receptors (RAR) with all-*trans* retinoic acid (ATRA) as the major ligand and the retinoid-X receptors (RXR) with 9-*cis* retinoic acid (9CRA) as a potential candidate ligand (13-15). The synthesis, degradation, cellular binding and receptor mediated binding of all-*trans* retinoic acid is tightly regulated (16).

Studies reported that vitamin A / retinoids modify the outcome and severity of atopic diseases, mainly the immune phenotype of these diseases (17-18). Skin specific KO-model of the nuclear retinoid receptors RAR and RXR and combinatory KO-mice models in skin display that retinoid signaling is highly important for the skin phenotype of AD (19-20). In this study we investigated genes known to be involved in retinoid regulation, metabolism, receptors and signaling of endogenous retinoids (Fig. 1) as well as retinoid target genes regarding their expression profile in skin from healthy volunteers as well as non-affected and affected skin from atopic dermatitis patients.

The aim of the study was to identify the expression profile from genes involved in retinoid metabolism, regulation and signaling as well as retinoid receptors and retinoid target genes in the skin. This was achieved by a comparable analysis of healthy and diseased skin biopsies and the additional consideration of serum as well

as skin retinoid concentrations via sensitive high performance liquid chromatography mass spectrometry – mass spectrometry (HPLC MS-MS) methodology.

Materials and methods

Skin biopsies and serum samples

Skin punch biopsy specimens were taken after obtaining informed consent in involved areas from 6 patients with atopic dermatitis (2 male and 4 female patients; average age, 31 years), according to the declaration of Helsinki, and from 6 non-atopic healthy volunteers (2 male and 4 female individuals; average age 30 years, see table 1) characterized by the absence of personal or family history of atopic disease. In case of AD patients, one biopsy of affected skin and one biopsy of non-affected skin were taken from each patient. Epidermis represents ca. 20-30 % of the skin biopsy used for analysis. Specimens were immediately frozen in dry ice and stored at -70 °C until RNA isolation or HPLC analysis was performed. At the same time, serum samples were drawn from the same patients and were kept at -70°C until analysis. Ethical approval for the study was obtained from the local ethics committee (EA1/168/06) and from each volunteer a signed informed consent was obtained.

Analysis of mRNA expression

Skin samples were homogenized in Tri® reagent solution and total RNA was isolated according to the manufacturer's guidelines. The concentration and purity of RNA were measured by means of NanoDrop spectrophotometer (Thermo) and its quality was checked using agarose-gel-electrophoresis. For real-time quantitative PCR (QRT-PCR), total RNA was reverse transcribed into cDNA using the Super Script II First-Standard Synthesis System (Invitrogen). QRT-PCR was carried out in triplicate using pre-designed MGB assays ordered from Applied Biosystems, on an ABI Prism 7900. Relative mRNA levels were calculated using either the comparative C_T or standard curve methods normalized to cyclophilin A mRNA. Sequence Detector Software (version 2.1) was utilized for data analysis and relative fold induction was determined by the comparative threshold cycle method.

High performance liquid chromatography mass spectrometry – mass spectrometry (HPLC MS-MS) analysis:

Concentrations of retinol and retinoic acids (Ras) were determined in human serum and skin biopsies by our HPLC-MS-MS method (21). In summary, 100 mg of the skin biopsy (if samples were under 100 mg water was added up to the used standard weight: 100 mg) or 100 µl serum was diluted with a threefold volume of isopropanol, the tissues were minced by scissors, vortexed for 10 seconds, put in a ultra sonic bath for 5 minutes, shaken for 6 minutes and centrifuged at 13000 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 µl of methanol, vortexed, shaked, diluted with 40 µl of 60 mM aqueous ammonium acetate solution and transferred into the autosampler and subsequently analysed.

Statistics

Data are shown as mean and standard error mean values of three measurements per data point. Statistical analysis was performed using the program SPSS 16.0. A *p* value of less than 0.05 was considered significant.

Results

A. Expression profiles of retinoid signalling pathways in AD skin samples

Firstly we investigated the expression profile of genes, which are involved in retinoid-homeostasis, -regulation, - metabolism, retinoid receptors and retinoid-target genes. These data show that there is a severe dysregulation of retinoid-homeostasis, -metabolism and -signalling present in affected and non-affected AD skin.

Dysregulation of retinol homeostasis / retinyl ester synthesis regulation

Retinol binding protein (RBP) 4 is the carrier protein involved in the transport of retinol and its mRNA expression was non-significantly down-regulated in atopic dermatitis affected skin (Fig. 2d). The mRNA expression of diacylglycerol acyltransferase (DGAT) showed a slight decrease in both affected and non-affected skin compared to healthy skin, while lecithin retinol acyltransferase (LRAT) was significantly up-regulated in non-affected skin of atopic dermatitis (Fig. 2a).

Dysregulation of retinal synthesis

Expression levels of beta - carotene 15,15`- monooxygenase (BCMO1) and beta-carotene-dioxygenase-2 (BCMO2) mRNA were not significantly altered in skin with atopic dermatitis compared to healthy skin (Fig. 2a). Retinol dehydrogenases and alcohol dehydrogenases are enzymes which are responsible for converting retinol to retinal. Retinol dehydrogenase (RDH) 2, RDH10 and RDH16 showed a very similar pattern in their mRNA expression. RDH2 was statistically significantly induced in both affected and non-affected atopic dermatitis skin, compared to healthy skin. Augmented mRNA expression of RDH10 and RDH16 was observed in atopic dermatitis affected skin, vs. healthy skin. The mRNA expression of alcohol dehydrogenase (ADH) 1C was significantly reduced in non-affected and affected AD skin (Fig. 2b).

CRBP 1 is the intracellular carrier of retinol and its mRNA expression showed a significant down-regulation in case of skin with atopic dermatitis, both in non-affected and affected AD skin (Fig. 2d).

Reduced retinoic acid synthesis in affected and non-affected skin of atopic dermatitis

Retinal dehydrogenase (RALDH) or acetaldehyde dehydrogenase converts retinaldehyde to RA. We observed a significant decrease of RALDH1 mRNA levels in skin with atopic disease, both in affected and non-affected skin, while the mRNA expression of RALDH2 and RALDH3 did not show significant alterations in our experimental setup (Fig. 2c).

CRABP2 is an intracellular retinoic acid transporter protein and its mRNA expression was not altered in skin of atopic dermatitis patients. Also CRABP1 did not show a significant alteration in diseased skin, in comparison to healthy skin (Fig. 2d).

Reduced expression of RA degradation / metabolism enzymes in affected as well as non-affected skin of atopic dermatitis

mRNA expression level of RA-degrading enzymes was determined and revealed a significant decrease in case of CYP26A1 and CYP2S1, both in affected and non-affected AD skin. However mRNA expression of CYP26B1 could not be detected in our experimental setup (Fig. 2e).

Increased expression of RXRα in non-affected skin of atopic dermatitis

RARα mRNA levels did not show any significant alteration in diseased compared to healthy skin, while mRNA expression of RARβ was slightly but non-significantly up-regulated in non-affected AD skin. By contrast, the expression of RARγ was comparable between atopic dermatitis and healthy skin. The expression of RXRα was significantly increased in non-affected AD skin and affected AD skin (Fig. 2f).

Expression of retinoid target genes in atopic dermatitis

The mRNA expression of retinoic acid receptor responder (RARRES1) was significantly decreased in case of atopic dermatitis in non-affected and affected skin, in comparison to healthy skin. Also TGM2 showed a significant decrease both in non-affected and in affected skin of atopic dermatitis. By contrast, HB-EGF mRNA expression was comparable between AD patients and healthy volunteers (Fig. 2g).

Due to strongly reduced retinoid signalling pathways in AD skin we next analysed ATRA and ROL concentrations in skin biopsies and serum of healthy volunteers and diseased patients:

B. Reduced concentrations of retinol and all-*trans* retinoic acid in AD skin, but not in serum of AD patients

Skin concentrations of ATRA and ROL were strongly reduced in affected (ATRA 0.4 / 0.5 ng/g; ROL 37 / 46 ng/g) but also in non-affected skin biopsies (ATRA 0.3 / 0.6 ng/g; ROL 32 / 54 ng/g) from an AD patient in comparison to ATRA and ROL concentrations in healthy skin (ATRA 0.7 / 1.2 ng/g; ROL 207 / 253 ng/g) (Fig. 3).

Serum concentrations of ATRA 2,8 ± 0,8 ng/ml and ROL 510 ± 217 ng/ml were comparable in healthy volunteers and AD patients ATRA 2,9 ± 1,0 ng/ml and ROL 573 ± 191 ng/ml (Table 2).

Discussion

In this study we demonstrated that in affected and in non-affected human tissue biopsies the retinoid transport, synthesis, concentrations, signaling and homeostasis are severely dysregulated in comparison to skin from healthy volunteers. To our surprise even the skin of non-affected areas of atopic dermatitis patients displayed dysfunction of retinoid signaling, suggesting an intrinsic disease specific dysfunction for the regulation of retinoid binding proteins, metabolizing enzymes, retinoid response target genes expression as well as retinoid concentrations. Interestingly the mRNA expression of the majority of retinoid response target genes like CRBP1, CYP26A1, CYP2S1, TGM2 and RARRES1 were significantly down-regulated, which is in accordance with the decreased level of retinoic acid determined in AD skin.

ATRA is the major RAR ligand and its concentration in the mammalian skin is tightly regulated in a specific spatiotemporal manner (19, 22-23). Various cell types in the skin and especially in the inflamed skin have been shown to be able to synthesize the bioactive retinoic acid (24). We found that retinoid response target genes like RARRES1, CRBP1, CYP26A1, CYP2S1 and TGM2 are significantly decreased in affected as well as non-affected human skin of AD patients, while the expression of other retinoid targets genes like RARβ, CRABP2 and HB-EGF were not altered. HPLC MS-MS data additionally confirmed that the concentration of ATRA is much lower in affected and in non-affected AD skin in comparison to skin from healthy volunteers; no difference was detected between affected and non-affected AD skin (Fig. 3). This might also be a cause or result of lower delivery of the retinoic acid precursor, retinol (ROL) via RBP4 to the skin, while both ROL levels as well as RBP4 expression are lower in affected AD skin samples in comparison to healthy volunteers (Fig. 2 and 3).

The additional analysis of serum concentrations from the same patients and volunteers displayed comparable ATRA levels between AD patients and healthy volunteers (Table 2). These data suggest a systemic non-RA based origin for this skin specific dysfunction of retinoid mediated signaling in AD. We suggest and partly know already that besides ATRA (the main signaling molecule for retinoid target

gene expression), which is much lower in skin of atopic patients, also other relevant and still non-identified bioactive retinoids or / and other retinoid mediated response pathways involving other retinoid-activated nuclear receptors must be present. Alternative activators of RAR and RXR may be responsible for stable and non-altered expression of the retinoid target genes RARβ, CRABP2 and HB-EGF in atopic skin even when ATRA levels are present in much lower concentrations. Identification of novel endogenous RAR as well as RXR ligands is under investigation in our laboratories.

The expression of the major retinoic acid synthesizing enzyme in the skin the RALDH1 is significantly decreased in AD skin vs. healthy skin (25). This strong down-regulation in affected and non-affected AD skin is suggested to be mainly responsible for lower ATRA concentrations and thereby for the significantly lower retinoid mediated signaling in the skin of AD patients.

Deficiency of retinoids / retinoid signaling in the skin or general vitamin A deficiency has been associated to various symptoms also seen in the atopic dermatitis skin phenotype. TH1 / TH2 shift (26), altered apoptosis (27), altered skin differentiation and proliferation (28) and increased bacterial skin colonization (29) were associated with vitamin A deficiency or deletion of retinoid receptor mediated signaling in transgenic skin specific mouse models (19-20). Whether lower retinoid signaling and lower retinoic acid concentration in AD skin is based on an intrinsic abnormality is under examination in various *in vivo* studies in our laboratories.

Remarkable is the reduced gene expression of the retinoid-target genes CRBP1, CYP26A1, CYP2S1, TGM2, RALDH1, RARRES1 and the ADH1C in non-affected AD skin comparably to affected AD skin. We suggest that a general and intrinsic abnormality is responsible for this dysregulation and maybe a result of systemic chronic inflammation. A different expression profile was observed for LRAT and RXRα which are exclusively increased in non-affected AD skin (Fig. 2) confirming also a general intrinsic abnormality responsible for this dysregulation of retinoid-signaling

(LRAT, RXRα) and maybe of other RXRα-heterodimer mediated pathways in non-affected AD skin. This increased expression of LRAT and RXRα maybe a response of the non-affected skin on intrinsic chronic inflammation to further enable and balance reduced retinoid signaling. Additionally the increased expression of RDH2 and RDH10 may be a skin based response to enable and balance retinoid signaling in the skin.

An altered nutrition with high vitamin A as well as pro-vitamin A carotenoids resulting in significantly higher serum levels of all-*trans* retinoic acid (30) or increased ingestion of dietary fats which lead to increased expression of various factors / enzymes important for retinoid signaling (31) might contribute also to this altered retinoid signaling in affected as well as non-affected skin of AD patients.

Several approaches using nutritional supplementations with carotenoids and various retinoids as well as systemic inflammation / allergic sensitization are in progress to elucidate why both in affected as well as in non-affected skin of atopic dermatitis patients retinoid transport, synthesis, concentrations and signaling are strongly decreased. We suggest that the answer to this question may help to understand the pathogenesis of atopic dermatitis and may lead to strategies for atopy prevention. Based on our observations we suggest that topical retinoid applications using single or combinations of selective retinoids would be highly beneficial for atopic dermatitis therapy.

In summary, more studies are needed to identify how retinoid transport, metabolism, concentrations and signaling are regulated in the skin and the regulation of key players like RALDH1, which is the major enzyme important for retinoic acid synthesis in human skin, in AD patients in comparison to healthy volunteers. Animal studies using topical as well as systemic application of various retinoids and KO animal models of retinoid synthesizing enzymes and retinoid receptors are in progress. We conclude that the retinoid signaling pathway is dysregulated in AD patients based on an abnormal retinoid transport, synthesis and concentrations which might contribute to the pathogenesis of AD, but also offer novel therapeutic approaches.

Acknowledgements

We thank Claudia Rasche for excellent technical assistance. The work is supported by the TAMOP 4.2.1./B-09/1/KONV-2010-007 project. The project is implemented through the New Hungary Development Plan, co-financed by the European Social fund.

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Table:

Table 1: Clinical and basic demographic data from the healthy and diseased donors

	Healthy volunteers	**AD patients**
age in years	30 ± 11	31 ± 14
gender	67 % female	67 % female
SCORRAD	0 ± 0	36 ± 11
total IgE (KU/L)	32 ± 17	1879 ± 764

Table 2: Concentrations of ATRA and ROL in ng/ml in serum of healthy volunteers (HEALTHY, n=6) and atopic dermatitis volunteers (AD, n=6). There were no significant differences between healthy volunteers and AD patients. For converting concentration into molar, 3 ng/ml ATRA and 2,86 ng/ml ROL represent a concentration of 10 nM.

	ATRA	**ROL**
HEALTHY	2,8 ± 0,8	510 ± 217
AD	2,9 ± 1,0	573 ± 191

Figure 1:

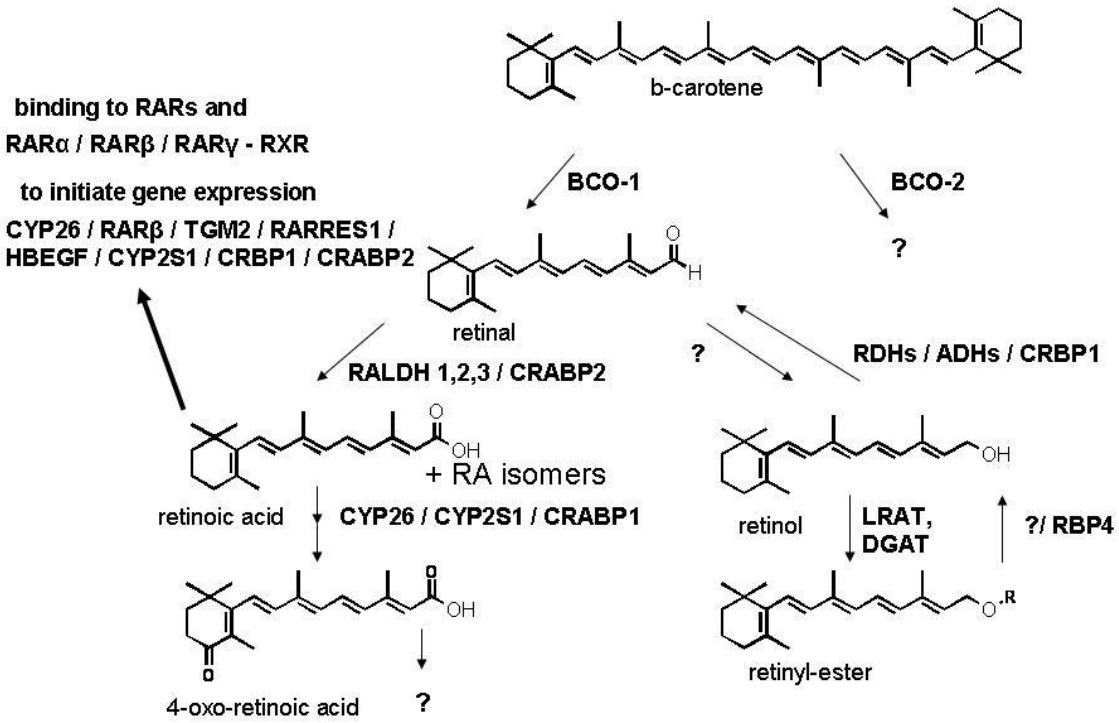
b-carotene
binding to RARs and
RARα / RARβ / RARγ - RXR
to initiate gene expression
CYP26 / RARβ / TGM2 / RARRES1 /
HBEGF / CYP2S1 / CRBP1 / CRABP2
BCO-1
BCO-2
?
retinal
RALDH 1,2,3 / CRABP2
?
RDHs / ADHs / CRBP1
+ RA isomers
retinoic acid
CYP26 / CYP2S1 / CRABP1
retinol
LRAT,
DGAT
?/ RBP4
4-oxo-retinoic acid
?
retinyl-ester

Figure 2:

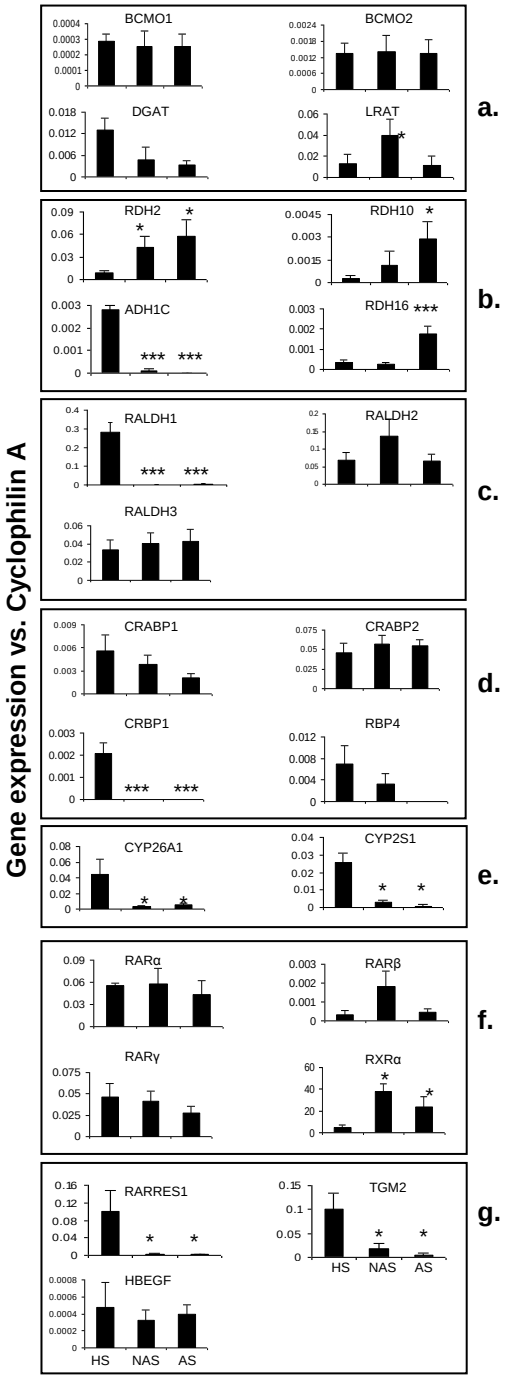
Gene expression vs. Cyclophilin A
BCMO1
BCMO2
DGAT
LRAT
a.
RDH2
RDH10
ADH1C
RDH16
b.
RALDH1
RALDH2
RALDH3
c.
CRABP1
CRABP2
CRBP1
RBP4
d.
CYP26A1
CYP2S1
e.
RARα
RARβ
RARγ
RXRα
f.
RARRES1
TGM2
HBEGF
HS
NAS
AS
g.

Figure 3:

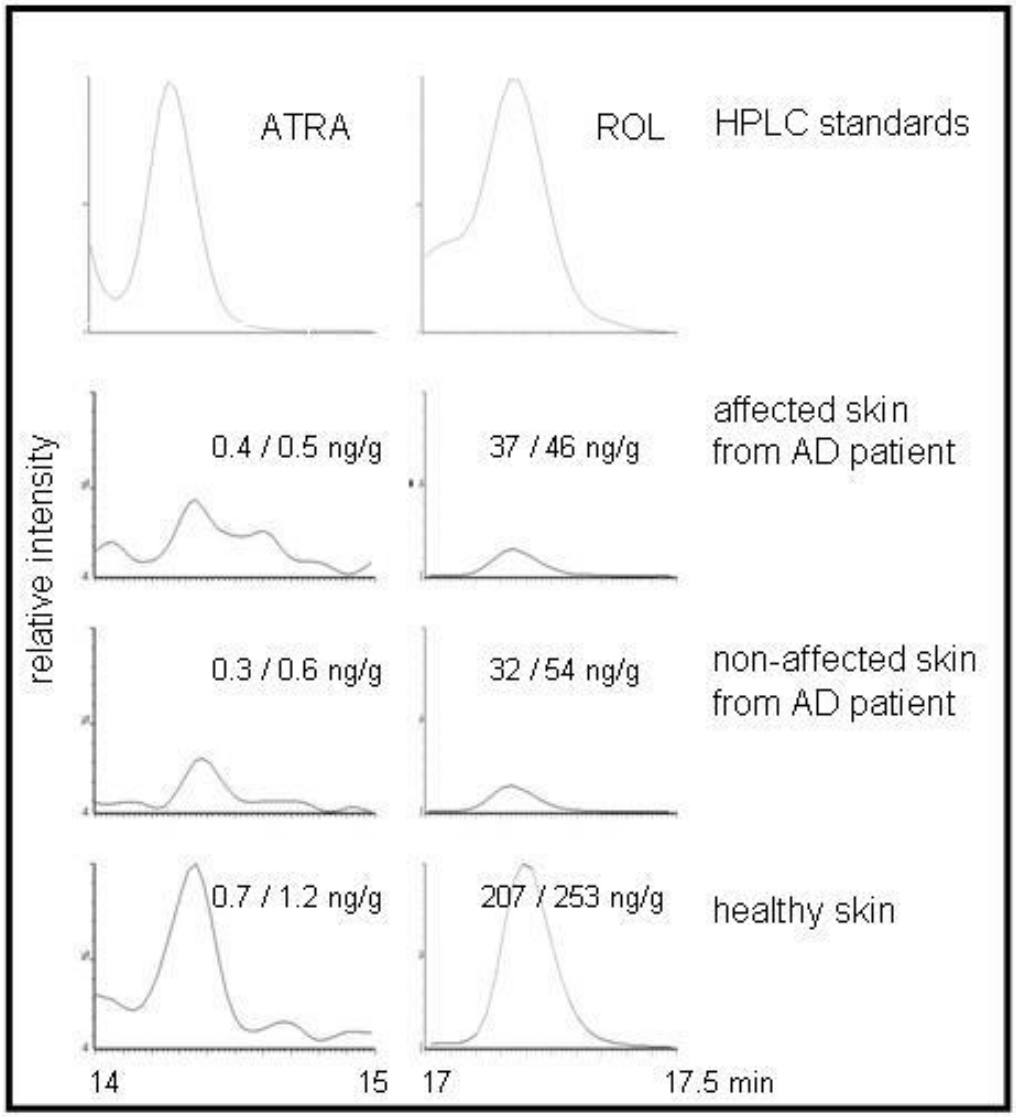
ATRA
ROL
HPLC standards
relative intensity
0.4 / 0.5 ng/g
37 / 46 ng/g
affected skin
from AD patient
0.3 / 0.6 ng/g
32 / 54 ng/g
non-affected skin
from AD patient
0.7 / 1.2 ng/g
207 / 253 ng/g
healthy skin
14
15
17
17.5 min

Figure legends:

Figure 1:

General scheme about retinoid signaling pathway including structures and involved binding proteins and retinoid-metabolizing enzymes: RA- retinoic acid, RDH – retinol dehydrogenase, RALDH – retinaldehyde dehydrogenase, BCMO - beta carotene oxygenase, CRBP - cellular retinol binding-protein, CRABP – cellular retinoic acid binding-protein, LRAT – lecithin retinol acyltransferase, DGAT – diacylglycerol acyltransferase, RBP4 – retinol binding protein.

Figure 2:

Expression profiles of genes involved in retinoid transport, metabolism and signaling pathway in healthy volunteers as well as in the skin of affected and non-affected AD-skin. HS – healthy skin, NAS – non-affected skin, atopic dermatitis, AS – affected skin, atopic dermatitis, * - $p < 0.05$, ** - $p < 0.01$, *** - $p < 0.001$

Figure 3:

HPLC MS-MS chromatogram of all-*trans* retinoic acid (ATRA) and retinol (ROL) using specific MS-MS tracks for ATRA and ROL from representative human skin biopsies of one affected and one non-affected skin biopsy from one AD-patient and one healthy volunteer. The two values represent the concentrations from the two independent determinations using the skin of two different healthy as well as AD-patients. Y-Axis of HPLC MS-MS chromatograms of the measured skin samples are the same magnitude for better visualization and comparison.