

SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Role of PPAR γ in human sebaceous gland biology and pathology

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The Examination takes place at Discussion Room, Department of Biochemistry and Molecular Biology, University of Debrecen, Faculty of Medicine, at 11 am, 8th of December, 2016.

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The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen at 13pm, 8th of December, 2016.

INTRODUCTION

1. Anatomy of sebaceous gland in human skin

Sebaceous glands (SG) are holocrine glands, built up of modified keratinocytes. SG two parts, glandular part (lobule or acinus) and duct. The gland consists of basal, dividing cells and mature, suprabasal and central sebocytes. The mature cells produce sebum continuously, as they synthesize lipids, these cells die, and the whole cells shed to the lumen of duct. Sebocyte turnover time estimated to be 14 days. The sebaceous gland duct is connected to a hair follicle mostly, and form pilosebaceous unit, rarely connected directly to the skin surface. Isthmus is part of hair follicle from the junction of sebaceous gland to the junction of apocrine sweat gland. Hair follicle from junction of sweat gland to epidermis is called infundibulum. Cells of hair shaft, which are in infundibulum shed to epidermis. The ductal cells, located under infundibulum, are called infrainfundibular cells, and shed to sebum.

2. Functions of sebaceous gland in human skin

SG produces sebum and multiple proteins. Sebum is a pale yellow viscous fluid composed of glycerides and free fatty acids (57%), squalenes (12%) wax and sterol esters (29%), and free sterols (2%). These lipids are called major lipids, as vast of sebum consist of these lipids. Depending on race, age of human, the quality and quantity of these lipids varies, but only in slight ranges. Sebum production at birth is relatively high, because of maternal hormones. In childhood it is low, and radically increase in puberty. Hormones, cytostatic drugs, anabolic steroids influence production of these lipids.

Sebocytes produce proteins, for example, androgen receptor (AR), fibroblast growth factor receptor (FGFR) , insulin – like growth factor (IGF-R). These play role in regulation of SG and surrounding cells. In addition, some molecules, like IL-1, IL-6 play role in inflammatory reactions as well and other pathological conditions of sebocytes.

3. Selected pathological conditions of sebaceous glands

Hereby I describe briefly the pathological conditions of SG, which we selected for the present research: sebaceous benign tumors (hyperplasia and adenoma), sebaceous carcinoma, and acne vulgaris.

Sebaceous hyperplasia is a common skin finding in aging adults, reported to occur almost 50% in newborns, and in adults, approximately 1% of the healthy US population, begin to appear in middle age (fifth decade). The hyperplastic glands are often inflated up to 10 times their normal size, they secrete very little sebum. Histology shows expansion of normal lobular sebaceous gland architecture without thickening of peripheral germinative layer of sebocytes. Sebaceous adenoma is rare tumor, presenting as a well-circumscribed flesh-colored or yellow papule or nodule, mainly located on the face, scalp or trunk. Histopathology shows nodular lobulated growth of cells in the deep dermis. The tumor consists of basaloid cells and sebaceous cells with cytoplasmic lipid vacuoles, but the lesion is not as organoid as sebaceous hyperplasia. Sebaceous carcinoma is also a rare tumour. The tumors presenting on skin has two forms: ocular (80%) and extra ocular (20%) (face, neck, genital area, trunk) localization. Seldom, it is localized extracutaneously, mainly in parotid gland. The tumor is soft, skin-colored, a few mm sized. Histopathology shows atypical epidermal cells with Pagetoid invasion in normal epidermis. Carcinoma cells are polygonal, diverse in morphology and form lobules in the dermis.

Acne vulgaris is a multifactorial disease, almost all people are affected during lifetime. Acne vulgaris appear as seborrhea, comedones (open or closed), hyperaemic papules, pustules or nodes, localized typically on surfaces which are rich in sebaceous glands. Acne vulgaris typically starts at puberty, as an androgen dependent process. Classical steps of acne formation consist of abnormal infundibular hyperkeratinization of sebaceous gland or follicular unit, elevated production of sebum, which stocks under the plug, and then a secondary inflammatory reaction is provoked, also *P. acnes* and other bacteria might have role in the process as well. In contrast,

some authors describe acne formation as a primary inflammatory, autoinflammatory disease, which is triggered by proinflammatory sebum lipid fractions. Pathomechanism of dermatoses involving SGs are complex. To develop better therapies, it is important to get more information about regulation of sebocytes.

4. Regulatory molecules of sebaceous glands

4.1 Hormones

Several hormonal regulation defects, which result in elevated androgen level, are accompanied by acne vulgaris. Also, elevated androgen receptor (AR) activity present in for example, congenital adrenal hyperplasia (CAH) syndrome, due to shorter CAG or GGN repeats in AR coding gene; also in raised gonadal or adrenal androgen production, or modification of androgen co regulators. In addition, puberty is the main age of acne, when androgens are produced in high level. Interestingly, first months of life some infants have acne, called 'acne infantum' or 'newborn acne', which is probably due to high androgen level of the mother. These hormones are primarily testosterone, ovarian and adrenal hormones. Interestingly, production of dihydrotestosterone in acneic skin is 30x higher than in normal skin.

Insulin-like hormones also have role in acne formation, as recent studies highlighted. Dietary factors with high glycaemic index like milk, and frequent sugar, cake and pastry intake were identified in mild and moderate acne compared to people with normal skin. Presence of acne in endocrinological changes with high serum insulin/ IGF-1 level like PCO and HAIR-AN syndromes also indicate high insulin level in acne. Recent research showed positive correlation with level of insulin like growth factor-1 and number of acne lesions in women and they also showed the possible molecular mechanism behind the phenomenon on SEB-1 human immortalized sebocytes. High dose insulin activated IGF-1, which attach and activates SREBP-1 pathway, which evolves lipogenesis in sebocytes.

Steroids, when administered in systemic and chronic course, also induce acne formation,

probably via the above-described pathway. Corticotrophin releasing hormone (CRH) / CRH-receptors are widely distributed in skin, and expressed on sebaceous glands. Excess of CRH is detected in acne involved SGs. CRH modulate inflammation as a proinflammatory molecule, in a direct paracrine effect on mast cells. In central nervous system, CRH is responsible for production of IL1, IL6, TNF- α . These cytokines also highly expressed in SG with acne vulgaris. Together with high expression of CRH, CRH-receptor 1 it suggests the role of CRH in acne evolution as well, as a regulatory molecule. Zouboulis C.C. suggested the theory „the brain of the skin: sebaceous gland” .

4.2 Cytokines

Alestars et. al. described that sebocytes produce cytokines: IL-6, IL-8, IL-1 β , and TNF α . They also investigated PG pathway (COX enzymes). PGE2, LTB4 molecules in acne pathomechanism, as in acne high enhancement of LT pathway, IL-6 and IL-8 cytokines was observed. Recently positive association was found between the minor T allele of the IL1A +4845(G>T) SNP and acne, and the severity of inflammatory acne symptoms correlated with the ratio of individuals carrying the homozygote T/T phenotype. Proinflammatory cytokines also play important role in acne pathogenesis. Two SNPs of TNF regulatory region have been detected recently, TNF α -857 minor T-allele was found to act as a protective factor in Hungarian study population in acne. In female acne higher occurrence of minor-308 A allele was found by Szabó et al. Toll like receptor (TLR) genes TLR2 and TLR4 are upregulated in KCs of acne lesions. In addition, Nagy et al. reported that distinct strains of *P. acnes* induced selective IL-8 and β -defensin-2 expressions in human keratinocytes through TLRs. IL1R and TLR has great structural homology, which might describe the IL-1 α - induced upregulation of TLR – mediated immune responses in acne vulgaris.

4.3 Transcription factors

Investigating the function of the sebaceous gland (SG) under normal and pathological conditions is a major area of research interest in dermatology. This includes investigations on the transcriptional mechanisms that regulate lipid metabolism and inflammatory responses of sebocytes. Identification of specific regulatory mechanisms and key actors in these processes is essential to fully understand the normal physiological function and to clarify the pathophysiological involvement of SGs in dermatoses. A particularly relevant area of research is the regulation of gene expression by transcription factors responding to changes in fatty molecules and in that way linking metabolic processes to gene regulation.

Many of the transcription factors, which play role in adipocytes, have role in SG cellular processes. Expression of C/EBP β , and C/EBP α was described in sebocytes in situ and in cultured SZ95 sebocytes as well. KLF5 mRNA and protein is expressed in sebocytes. Early B cell factors (EBFs) were not described in sebocytes, however, expression of an indirect target gene of EBFs SREBP-1 and many of steroidogenic enzymes were detected in sebocytes. A role of Melanocortin receptor 5 (MC5R) was demonstrated in lipid production in animal models. Its ligand, α -MSH, has a modulatory role in SG inflammation. PPARs are also important factors in sebaceous lipogenesis.

5. Peroxisome proliferator-activated receptor γ (PPAR γ)

5.1 Functions of PPAR γ molecule

The nuclear hormone receptor PPAR γ (NR1C3) was initially characterized as a master regulator of adipogenesis, and its firstly identified target gene was FABP4/aP2 gene. However, later several studies revealed, that PPAR γ is present and active in several other cell types, for example immune cells, skeletal muscle cells, gastrointestinal epithelial cells. Increasing number of cellular processes, apart from lipogenesis, in which PPAR γ plays role was described, like inflammation, atherosclerosis, bone morphogenesis and

cancer. In addition, PPAR γ is expressed in SGs. Furthermore, PPAR γ may be an important molecule in acne vulgaris, the most frequent SG-related dermatosis with abnormal lipid storage and inflammation. In addition, role of PPAR γ is observed in PCOS, a specific endocrinological syndrome, which involves skin with acne and hirsutism, often associated with type 2 diabetes and obesity, and shows high insulin and androgen serum level. Certain molecules work together with PPARs in SG. PPARs cooperate with LXR (liver X receptor), which was reported to regulate lipid metabolism in SZ95 cells. FoxO1 as a cosuppressor, antagonizes PPAR γ activity, and inhibits expression as well. Endocannabinoids via cannabinoid receptor 2 enhanced lipid synthesis and apoptosis of human sebocytes, which upregulated PPARs and PPAR mediated target genes.

5.2 Ligands for PPAR γ molecule

Ligand for PPARs are exogeneous, artificial compounds and endogenous, molecules which are produced by the human organ. Thiazolidiones are ligands of PPAR γ (troglitazone, pioglitazone, ciglitazone, englitazone) have antihyperglycaemic effect and insulin sensitizers on peripheral cells. Several NSAIDs also bind to PPAR α and γ , (indomethacin, fonoprofen, flufenamic acid, ibuprofen). However, the nature and identity of endogenous ligands that activate PPAR γ in the skin are not known. Potential ligands of PPARs include fatty acids, various eicosanoids, and other lipids found in the skin and also in SGs. Previous studies have shown that AA can activate PPAR γ ; however, it is highly likely that not AA, but some of its metabolites are the main bioactive mediators of PPAR γ activation.

Taken together these findings prompted us to systematically assess the potential role of PPAR γ in human sebocytes. We also sought to identify endogenous PPAR γ ligands among AA derivatives, which activate the receptor, along with gathering evidence on

target gene expression and roles in differentiation and lipid metabolism both in situ and in vitro.

To address these issues, including the expression levels of PPARs and the presence of PPAR γ in normal and pathological conditions, we have examined the expression of this transcription factor in sebocytes in two different human SG model systems: i) in laser-micro-dissected SG from fresh frozen tissues; and ii) in a human immortalized SG cell line, namely SZ95. We also determined PPAR γ expression pattern in sebaceous hyperplasia, adenoma and carcinoma tissues. We found that AA and AA-derived keto-metabolites apparently modulate PPAR γ activity, which appears to be important in the regulation of SG differentiation and lipid biosynthesis.

AIMS

Our study focused on the role of transcriptional factor PPAR γ in human sebaceous glands. As more specific goal, we aimed to:

- 1) Detect PPAR γ and differentiation markers of sebocytes in normal and pathological sebaceous glands,
- 2) Identify PPAR γ and markers of its activity in sebaceous glands in situ and in vitro,
- 3) Identify endogenous ligands of PPAR γ in sebaceous glands ,
- 4) Find role of PPAR γ in sebocyte lipid production, characterize the PPAR γ activation induced changes in lipid profile of sebaceous glands,
- 5) Define PPAR γ expression and its ligands in sebaceous glands of patients with acne vulgaris (because this is the most common, SG involved disease, which affects life-quality).

MATERIALS AND METHODS

1. Cell culturing

Human immortalized sebocytes SZ95 (Zouboulis et al., 1999) were cultured in Sebomed basal medium (Biochrom, Berlin, Germany) supplemented with 10% FBS (Invitrogen, Carlsbad, CA, USA), 5 ng/ml epidermal growth factor (Sigma, St. Louis, MO, USA), 50 U/ml penicillin and 50 U/ml streptomycin (both from Biogal, Debrecen, Hungary) and 1 mM CaCl₂. The medium was changed every other day and cells were sub-cultured at 80-90% confluence.

2. Determination of intracellular lipids

SZ95 sebocytes washed in phosphate-buffered saline (PBS), and stained with 1 µg/ml Nile red (Sigma-Aldrich) solution in PBS for 20 minutes at 37 °C and the fluorescence released was determined by fluorescence microscope (Leica microsystems). For quantitative measurement Molecular Devices FlexStation384 II Fluorescence Image Microplate Reader (FLIPR, Molecular Devices, San Francisco, CA USA) in Physiology Department of University of Debrecen was used.

3. Determination of membrane lipid species by ESI-MS/MS

Quantitative measurement of membrane lipid classes in SZ95 sebocytes was carried out by Gerhard Liebisch and Gerd Schmitz (Klinische Chemie und Laboratoriumsmedizin, University Hospital Regensburg, Germany), by direct flow injection electrospray ionization tandem mass spectrometry (ESI-MS/MS) in positive ion mode using the analytical setup and strategy.

4. Detection of major neutral lipid classes

SZ95 sebocyte major neutral lipid classes were measured by Mauro Picardo and Emmanuela Camera, (Istituto Dermatologico San Gallico, Rome Italy), by separating crude lipid extracts with high performance thin layer chromatography, time of flight mode mass

spectrometry (HPTLC ToF/MS).

5. Determination of eicosanoids / docosanoids using HPLC-MS-MS methodology

For quantitative determination of eicosanoids and docosanoids in SZ95 cells, the HPLC-MS-MS methodology was used, by Ralph Rühl (UD, Department of Biochemistry and Molecular Biology) .

6. RNA isolation, Real Time –quantitative PCR (RT-qPCR)

Total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacture's protocol. Reverse transcription was performed at 42°C for 30 min from 100ng of total RNA using Superscript II reverse transcriptase (Invitrogene, Carlsbad, CA, USA). Quantitative PCR was performed on ABI PRISM 7900 instrument (Applied Biosystems Foster City, CA, USA). The comparative Ct method was used to quantify transcripts and normalize for human cyclophilin A.

For LCM derived samples the RT and qPCR reactions were made in one single step using the Cells Direct One Step RT-qPCR kit (Invitrogene, Cat. No: 11754-100, Carlsbad, CA, USA), according to the manufacturer's instruction.

7. Statistical analysis

All data are presented as mean \pm SD. When applicable, data were analyzed using a two tailed un-paired t-test and $P < 0.05$ values were regarded as significant differences. In addition, statistical differences were further verified using 1-way ANOVA with Bonferroni and Dunnett post hoc probes, resulting in similar results.

8. Ligands

Cells were treated with the following ligands: PPAR γ selective agonist rosiglitazone (RSG), antagonist GW9662 (Alexis Biochemicals), RXR panagonist LG100268, a gift

from R. Heyman (Ligand Pharmaceuticals), arachidonic acid (Sigma), 5-KETE, 12-KETE, 15-KETE. (Cayman Biochemicals).

9. Patient samples

The study was approved by the Institutional Research Ethics Committee and adhered to Declaration of Helsinki Guidelines. Formalin-fixed and paraffin embedded tumor tissues were obtained from the archive files of Departments of Dermatology and Pathology, University of Debrecen. The human tissue samples were all handled as anonymous in respect of patients' dignity.

Patients with acne vulgaris were examined according to the Global Acne Grading System Score (GAGS). The biopsies were obtained from the upper back sites, non-acne involved skin with acne vulgaris, and acne-involved skin samples on acneic papules from the same donors, and stored in -80°C . For further experiments cryosections were used. Skin of healthy donors as reference were used. Two samples with sebaceous adenoma and sebaceous hyperplasia were cut to two pieces. One part was formalin fixed and the other was stored in -80°C , and cryosections were used for further experiments.

10. Immunohistochemistry (IHC) and double immunofluorescence (IF)

IHC for PPAR γ was carried out using the biotin-free and tyramid-based CSAII kit (Dako, Glostrup, Denmark) according to the manufacturer's instruction. For immunocytochemistry sebocytes were cultured on coverslip slides, and fixed with 1% paraformaldehyde. Single IHC stainings for androgen receptor (AR), and p63, Ki67 were detected with standard immune-peroxidase method using an EnVision+-HRP (horse-raddish-peroxidase) detection kit or an automated immune-stainer (Bond-Leica, UK). The following antibodies were used: PPAR γ (Santa-Cruz, E8; 1:75), mab-AR and mab-p63 both from Novocastra (Newcastle, UK), ki67 (1:30; clone EPR3611, BioGenex, San Ramon, CA, USA) according to vendor's instructions. The immunostained slides were then

evaluated semi-quantitatively for sebaceous glands/SG tumors, reflecting the % of positive cells: -, negative; 1+, 0-20%; 2+, 20-50%; 3+, 50-75%; 4+, 75-100%. When double IHC immune-peroxidase staining for the simultaneous detection of PPAR γ and Ki67 was made, a standard sequential labelling method was applied. The nuclear counterstaining was with methyl-green or Meyers hematoxylin.

Double IF detection was carried out by Prof. dr. Balázs Dezső (UD, Department of Pathology), using TSA-TMR/FITC systems (Perkin Elemer, USA), according to protocols provided by the vendor. For IF, the nuclear counterstaining was made with DAPI.

11. Laser Capture Microdissection (LCM) and RNA preparation

For each specimen, serial sections were made in a cryo-microtome machine (Leica) to collect 12 x 5 μ m thick identical sections mounted onto the polyethylene slide-membrane (Olympus Europe and Molecular Machines and Industries, Hamburg, Germany) for LCM followed by an immediate dehydration and fixation in alcohol at room temperature using nuclease free water for the diluent of 70% ethanol. The region of interest was cut by the computer-guided laser beam MMI-Olympus Cellcut Laser Capture Microdissection system (Olympus Europe and Molecular Machines and Industries, Hamburg, Germany) and transferred to the lifting cap of the sterile RNase-free eppendorf tube to collect the samples for molecular biology analyses. RNA was extracted by Absolutely RNA Nanoprep kit (cat. No: 400753, Stratagene, La Jolla, CA, USA) according to the manufacture's instructions. Samples were stored on -70°C for long-term usage.

12. Detection of viable cells

For quantitative measurement of viability, MTT(3- (4,5- dimethylthiazol) 2,5-diphenyl tetrazolium bromide) assay was carried out as described previously by Bodó et al.

RESULTS

1. PPAR γ and its target genes are expressed in normal human SGs in situ

First, we determined the expression level of PPAR γ and markers of its activity in human SGs in situ. Immuno-labeling identified PPAR γ protein expression predominantly in the nuclei of normal SGs of skin biopsies obtained from healthy volunteers (n=10). In addition, SGs were isolated from donors of healthy (n=5) and pathologic (n=2) human skin tissues by laser-microdissection and mRNA levels of PPAR γ were examined by quantitative real-time PCR (qPCR). The expression of PPAR γ mRNA was higher in healthy SG than in pathologic samples, although the latter was available only in limited numbers.

It is generally accepted that PPAR γ activity is indicated by the mRNA expression of its target genes including the adipose differentiation related protein (ADRP) and PPAR γ angioprotein related protein (PGAR), as established in adipocytes, macrophages and dendritic cells in previous studies. We also found that laser-microdissected SG samples from healthy individuals express ADRP and PGAR mRNA at higher levels than SGs from pathologies. These data suggest that PPAR γ and also its target genes are present in human SGs.

2. Active PPAR γ is present in human SZ95 sebocytes

Expressions of the three PPAR isoforms were detected at the mRNA level in SZ95 sebocytes. Next, we investigated PPAR γ activity in SZ95 sebocytes. We treated SZ95 sebocytes with the synthetic PPAR γ agonist rosiglitazone (RSG) and measured the mRNA levels of two target genes, PGAR and ADRP. RSG (1-10 μ M) induced significant upregulation of PGAR and ADRP. Administration of a PPAR γ inhibitor GW9662 markedly abrogated the RSG-induced target gene induction in a dose-dependent manner (1-10 μ M). These data collectively suggest that PPAR γ is active in human SZ95 sebocytes.

3. PPAR γ expression correlates with the differentiation stage of sebocytes

Next we sought to define the role of PPAR γ in the process of sebocyte differentiation. Therefore, we investigated SGs from human biopsies obtained from patients with benign or malignant SG lesions. Immunohistochemical analyses revealed the nuclear expression of PPAR γ in normal SGs. Remarkably, PPAR γ protein was hardly detectable in basal cell layers of SG (n=10) in immature (non-differentiated) sebocytes. PPAR γ was present at high levels in sebocytes of all samples (n=10) from patients with SG hyperplasia. PPAR γ was detectable in terminally differentiated mature sebocytes located in the central regions of normal SGs, but this protein was hardly detectable in immature cells of the basal layer. We also analyzed samples of SG-tumors. When compared to normal and hyperplastic SGs, cells of sebaceous adenomas (n=10) generally expressed decreased amounts of PPAR γ , which appeared to decrease further in malignant tissues and sebaceous carcinomas (n=9) and down to hardly detectable levels in poorly differentiated neoplastic cells. This was confirmed by using established markers of cell cycle activity and differentiation such as Ki67, p63 and AR molecules.

We then corroborated our results by investigating the PPAR γ expression pattern and its signaling pathway during normal sebocyte differentiation. Therefore, terminally differentiated normal SG cells (i.e., from the central zone of the gland) and immature cells (from the basal-parabasal zones) were collected separately by in situ laser microdissection from fresh frozen human skin tissues; all samples were from the facial region of 5 different donors. PPAR γ , ADRP and PGAR were expressed at low levels in basal cells, whereas higher expressions were detected in the well-differentiated sebocytes of the central zone. These data together with findings from the 10 samples analyzed with immunohistochemistry indicated that PPAR γ may be employed as a potential marker of sebocyte differentiation.

4. AA regulates sebocyte differentiation, in part via PPAR γ -coupled signaling pathways

To further explore the biological consequences of PPAR γ stimulation on SG biology, we also investigated the functional role of PPAR γ in SZ95 sebocytes. Sebocyte differentiation is characterized by the synthesis of various lipids, among which neutral lipids represent the major part. Confirming our previous results, first we showed that AA, one of the most effective differentiation inducer of SZ95 sebocytes, markedly enhanced lipid synthesis of the cells. Quantitative Nile red-based fluorescence assay was performed, which showed that AA induced neutral lipogenesis in SZ95 sebocytes in a dose-dependent manner. Intriguingly, the PPAR γ agonist RSG also induced sebaceous neutral lipid synthesis in SZ95 sebocytes. Also, AA treatment of the cells resulted in a marked upregulation of the mRNA expression levels of the PPAR γ target genes (PGAR and ADRP) which effect could be fully abrogated by a PPAR γ specific antagonist GW9662. Moreover, the lipid synthesis-promoting activity of AA was also inhibited by a PPAR γ antagonist. However, it must be noted that GW9662 was able to only partially (albeit significantly) prevent the effect of AA. Finally, we found that AA treatment also upregulated the mRNA level of PPAR γ .

5. AA regulates the production of selected lipid molecules via PPAR γ

To further explore how PPAR γ activation affects lipid production in sebocytes, we treated the cells with AA (25 μ M) and with a PPAR γ antagonist GW9662 (10 μ M) simultaneously for 24h and then analyzed the major neutral lipids and specific membrane lipids. We determined the relative abundance of major neutral lipid classes, such as TG, FFA, CE, CH, WE, and SQ using high performance thin layer chromatography (HPTLC) followed by densitometric analyses. Quantitative determinations of AA were performed by high pressure liquid chromatography coupled with tandem mass spectrometry (HPLC-QqQ/MS), whereas concentrations of SQ and CH and the relative abundance of AA-

induced DG and TG were determined by HPLC-ToF/MS.

HPTLC of lipid extracts of cell pellets resulted in the detection of cholesterol (CH), free fatty acid (FFA), triglycerides (TG), wax esters (WE) and cholesterol esters (CE). Squalene (SQ) and diacyl glycerol (DG) were undetectable under these analytical conditions. Densitometric analyses of HPTLC spots showed that TG levels were significantly elevated by AA and this induction was inhibited by a PPAR γ inhibitor. Substrate concentration of AA was determined quantitatively and exposing sebocytes to AA resulted in a significant elevation of AA concentration in the cells, while co-administration of GW9662 appeared not to influence these cellular levels of free AA.

Biosynthesis of TG is a multistep process that involves different lipid intermediates. To gain insight into the mechanisms involving PPAR γ activation, we determined the relative abundance of AA-induced DG and TG by HPLC-MS analyses. The AA containing DG 40:8 was significantly induced by AA, while GW9662 did not influence DG 40:8 levels. The levels of the TG 60:12, representative of the AA-containing TG were induced to a significant extent.

When AA was co-administered with a PPAR γ antagonist this induction of TG 60:12 was inhibited, which is consistently with the HPTLC results concerning the total TG. Also, AA induced WE levels, however no effect of the PPAR γ antagonist on this was observed. Additionally, AA had no significant effect on the total CE levels regardless of the presence of the PPAR γ antagonist. Interestingly, basal CH level was significantly decreased by the PPAR γ antagonist GW9662 as demonstrated by the quantitative analysis of CH by HPLC-MS. In contrast, concentration of SQ determined by HPLC-MS was not affected by AA alone or in combination with the PPAR γ antagonist GW9662.

Next, using direct flow injection electrospray ionization tandem mass

spectrometry (ESI-MS/MS), nine main specific membrane lipid groups were assessed: phosphatidylinositol, lysophosphatidylcholine, sphingomyelin, dihydrosphingomyelin, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, ceramide and glucosylceramide. AA had no significant effect on ceramides and glucosylceramides. Among the other 7 measured membrane lipid groups, AA induced significant alterations in the levels of 136 of the 198 measured lipid species. Importantly, the presence of the PPAR γ inhibitor GW9662 significantly prevented the action of AA on 8 lipid species. Among these molecules, AA decreased the level of seven types of phosphatidylinositol (PI) whereas it upregulated the expression of 18:2 lysophosphatidylcholine. These findings suggest that AA regulates the metabolism of these molecules in a PPAR γ dependent fashion.

Therefore, the AA-coupled PPAR γ pathway may contribute to the generation of major neutral lipids and membrane phospholipid production. With this we comprehensively analyzed lipid changes upon AA treatment, and sorted out the ones requiring PPAR γ activation.

6. AA metabolites activate PPAR γ and induce lipogenesis in sebocytes

AA can be metabolized to a plethora of signal lipids. Therefore, we assessed whether certain AA metabolites can also induce similar cellular responses on human sebocytes. SZ95 sebocytes were treated with AA for 6 hrs and an HPLC-MS approach was then employed to quantitatively determine the levels of AA-derived signalling lipids. Upon AA treatment, we detected increased concentrations of 5-KETE, 12-KETE, and 15-KETE. Furthermore, these endogenous AA metabolites significantly elevated sebaceous lipid synthesis and significantly increased the gene expression levels of PGAR and ADRP. However, the PPAR γ antagonist GW9662 was able to inhibit the induction of PGAR only in the case of 12-KETE and 5-KETE, and the induction of ADRP in the

case of 12-KETE. Taken together, these findings indicate that AA-metabolites play a role in sebaceous lipogenesis and that 12-KETE and 5-KETE are likely to signal via PPAR γ .

7. PPAR γ and its target genes are expressed in situ both in normal SG, and in the non- acne-involved and acne-involved SGs of acne patients

Immunolabeling identified PPAR γ protein expression predominantly in the nuclei of normal SGs obtained from healthy volunteers (3+), but low expression was detected in SG cells of acne patients, both in non-acne-involved (NAS) and in acne involved skin (AS) samples as well. The expression of PPAR γ , ADRP, PGAR mRNA was higher in SGs from healthy individuals, than in NAS and AS samples, respectively. These data suggest that PPAR γ and its target genes are present in healthy SGs of acne patients and also in SGs of age matched healthy donors.

We determined the level of various PPAR γ -activating eicosanoids in skin biopsies from patients. The level of PPAR γ -activating eicosanoids was lowest in healthy skin samples, while NAS-acne skin samples had higher and AS-acne samples had the highest level of eicosanoids. This might suggest, that the level of PPAR γ -activating eicosanoids are sufficient or even present in excess in SG of acne patients, maybe due to the low level of PPAR γ and feedback regulation in SG samples from acne patients.

DISCUSSION

Previous studies have shown that PPAR γ is expressed in normal SG, in primary sebocytes and in SZ95 sebocytes. However, very little is known on the expression level of this important transcription factor in situ. We intended to extend these studies by using laser microdissection. We found that less differentiated sebocytes express lower levels of PPAR γ and its target genes, as compared to more differentiated sebocytes. This was confirmed by using established markers of cell cycle activity and differentiation such as Ki67, p63 and AR molecules. We also compared the expression of these molecules in sebaceous hyperplasia and found that the expressions of PPAR γ and the other molecules detected were essentially the same as detected in normal SG. In contrast, the proportion of cells expressing PPAR γ in sebaceous adenoma and sebaceous carcinoma is lower, but the proportion of Ki67, and p63 positive cells is higher. Recently, Cottle et al. described the expression of AR (basal, parabasal layer), Ki67 (some in basal layer) and PPAR γ (intermediate, i.e. outer central layer) in healthy SG of murine origin and gathered similar findings with AR in SG tumors. Additionally, the differentiation stage of carcinomas appeared to be correlated with the presence of PPAR γ in tumor cells. This suggests that PPAR γ might be a reliable diagnostic marker of sebocyte differentiation state. In addition, we detected that PPAR γ and its target genes ADRP and PGAR were expressed lower in sebocytes of AS from acne patients compared to sebocytes from healthy individuals. Interestingly, the receptor and its targets were detected at low level in non-acne involved SG from acne patients. These results appear to be consistent with the established anti-inflammatory role of PPAR γ observed on inflammatory bowel disease and also observed in lichen planopilaris and indicate that the inflammatory milieu is likely to contribute to reduced receptor levels and signaling.

In SZ95 sebocytes, one of the best available model systems for assessing human

sebocyte function, we confirmed our previous results based on the observation that PPAR γ mediated the expression of ADRP and PGAR target genes. Using a model of AA-treated differentiated sebocytes, we observed that the lipid rich SZ95 sebocytes expressed high levels of PPAR γ and also its target genes PGAR and ADRP, consistent with our previous finding, where the AEA (endocannabinoid) induced differentiation/lipid production was shown to be induced via PPAR γ . These data collectively suggest that PPAR γ is active in human SZ95 sebocytes.

Prior research has shown that RSG-activated PPAR γ has a role in sebaceous lipid production, a marker of sebocyte differentiation. In our study both the natural ligand precursor, AA, and the synthetic ligand, RSG, induced sebaceous lipogenesis similar to previous results. In addition, we offer pharmacological evidence indicating that the specific PPAR γ antagonist GW9662 dose - dependently inhibited this AA mediated target gene induction. This suggests that AA/ or derivatives activated PPAR γ -induced gene activation coincides with sebocytes' neutral lipid production. Therefore, we analyzed neutral lipids, which are the major components of the sebaceous lipid mixture, along with membrane lipid species. We observed that TG levels were significantly elevated by AA, and this effect was inhibited by PPAR γ antagonist by ca. 25%. The PPAR γ inhibitor GW9662 also lowered the basal level of CH; however AA did not have an effect on cholesterol level. It is likely that, under our experimental conditions, preformed AA caused remodeling of lipid species bearing FA side chains, such as GLP and TG, and affected minimally the non-esterified lipid components. Taken together these data suggest that TG and cholesterol production is likely to be regulated by PPAR γ in sebocytes, however AA (in the dose we used) had no effect on cholesterol levels. It is also likely that pathways leading to neutral lipid formation were regulated by AA in a selective manner.

In addition, we found that AA treatment also causes PPAR γ dependent elevation of some minor lipids, like LPC and PIs. In sebocytes, LPC is a minor phospholipid in membranes of lipid droplets consist of monolayer phospholipids. Lipid droplets in a wide range of cell lines (i.e. adipocytes, keratinocytes) were shown to be involved both in deposition and storage of neutral lipids, and were shown to have ADRP protein on their surface. These data might indicate that PPAR γ is involved in the formation of these lipid droplets, i.e. neutral lipid storage or production.

AA also significantly decreased the level of PIs in a PPAR γ -dependent manner suggesting that enzymes, which are involved in the synthesis/degradation of PIs are also influenced by the nuclear receptor activation. Moreover phosphoinositides, are key signal transduction molecules. The IP3 kinase pathway has a common part with the MAPK induced pathway, which was shown to be involved in AEA induced sebaceous lipid synthesis, in which PPAR γ also played a role.

Phospholipase A2 (PLA2) is the enzyme type responsible for hydrolyzing fatty acids like AA from glycerophospholipids. Therefore they represent the source for the AA production. PLA2 was detected in various cells of skin and also in SZ95 sebocytes. In addition it was shown, that treatment with active PLA2 increased neutral and polar lipid production of sebocytes. Therefore, it seems possible, that PLA2 is one regulator of endogenous AA production in sebocytes, and it also determines the level of PPAR γ activation in these cells at least in part. The identification of endogenous AA metabolites (such as 5-KETE, 12-KETE) achieved in this study supports this scenario. In addition, the reduction of the PIs level also suggests that there is a feedback mechanism aimed at the regulation of AA production if it exogenously added.

Although PPAR γ probably controls both the levels of major neutral lipids and level of LPC and PIs, the exact mechanism of action in sebaceous differentiation remains

to be discovered.

Another key goal of our study was to identify novel endogenous ligands, which might activate PPAR γ in sebocytes. Here, we show that the AA keto-metabolites (5-KETE and 12-KETE) act as endogenous PPAR γ ligands in SZ95 sebocytes as they elevated sebaceous lipid synthesis in a PPAR γ -dependent manner. Our data demonstrated that the same compounds are present in acne affected skin and their levels are increased. Therefore, it is tempting to speculate that this might be the part of a feed back regulation. However, other lipids or cytokines might contribute to regulation as well. Nevertheless, our findings suggest that PPAR γ might play a protective role in normal human sebocytes against excessive lipid accumulation and induction of inflammatory responses. In the skin of acne patients, PPAR γ is lower or even absent in inflamed sebaceous glands, which appear to be a major feature and maybe the reason of disregulated lipid homeostasis and inflammation. Confirming this theory, it is known that topical application of the PPAR γ agonist Azelaic acid transmits anti-inflammatory effects in acne vulgaris, which also supports the possible anti-inflammatory role of PPAR γ in sebocytes.

SUMMARY

SG produces 80% of lipids on the surface of the skin. These lipids play major role in epidermal barrier. Therefore it is a key question in skin research, how SG is regulated. In my work presented here, it is clearly shown, that PPAR γ is differentially expressed in normal and pathological human sebocytes, and appears to have roles in their differentiation and lipid production.

PPAR γ and its target genes, ADRP and PGAR, expressed in sebocytes and shown association with SC differentiation. PPAR γ is also present in normal and hyperplastic SG, whereas its expression levels were decreased in cases obtained from SG adenoma and SG carcinoma cells, reflecting a maturation-linked expression pattern. Our data also clearly show that PPAR γ levels and signaling activity in sebocytes from acne patients is decreased. Furthermore, in SZ95 sebocytes, naturally occurring lipids including arachidonic acid and arachidonic acid keto-metabolites (e.g. 5-KETE, 12-KETE) appear to regulate PPAR γ signaling pathways, which in turn modulate phospholipid biosynthesis and induce neutral lipid synthesis (Figure 17.). Collectively, our findings highlight the importance of endogenous ligand-activated PPAR γ signaling in human sebocyte biology and suggest that PPAR γ might be a promising candidate for the clinical management of SG disorders.

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List of publications related to the dissertation

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