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PPARγ-mediated and arachidonic acid-dependent signaling is involved in differentiation and lipid production of human sebocytes

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Abbreviations:

AA: arachidonic acid (eicosatetraenoic acid, 20:4)
ADRP: adipose differentiation related protein
CE: cholesterol esters
CH : cholesterol
CER: ceramides
DG: diglycerides
FFA: free fatty acids
GLP: glycerophospholipids
HE: hematoxylin-eosin stain
IHC: immunohistochemistry
ISTD: internal standard
LCM: laser capture microdissection
LPC: lysophosphatidylcholine
MQ water: milli-Q water
PC: phosphatidylcholine
PE: phosphatidylethanolamine
PI: phosphatidylinositol
PGAR: PPARgamma angiopoietin related protein
PPAR: peroxisome proliferator-activated receptor
qPCR: quantitative polymerase chain reaction
PS : phosphatidylserine
PG: phosphatidylglycerol
Plasm: PE-based plasmalogens
RT-qPCR: Real Time –quantitative PCR
RSG: rosiglitazone
RXR: retinoid X receptor
SM: sphingomyelin
SG: sebaceous gland
SQ: squalene
TG: triglycerides
WE: wax esters
5-KETE : 5-oxo-6E,8Z,11Z,14Z-eicosatetraenoic acid
15-KETE: 15-oxo-5Z,8Z,11Z,13E-eicosatetraenoic acid
12-KETE: 12-oxo-5Z,8Z,10E,14Z-eicosatetraenoic acid

ABSTRACT

The transcriptional basis of sebocyte differentiation and lipid production is mostly unclear. Peroxisome proliferator-activated receptor gamma (PPARγ), a lipid activated transcription factor, has been implicated in differentiation and lipid metabolism of various cell types. Here, we show that PPARγ is differentially expressed in normal and pathological human sebocytes, and appears to have roles in their differentiation and lipid production. We used laser microdissected normal and pathologic human sebaceous glands (SG) and SZ95 cells (immortalized sebocyte cell line) analyzed by RT-qPCR and immunohistochemistry. Lipids were analyzed by quantitative fluorimetry and mass spectrometry based approaches. We have observed that PPARγ and its target genes, ADRP and PGAR, are expressed in sebocytes and show association with their level of differentiation. Also, PPARγ is present in normal and hyperplastic SG, whereas its expression levels are decreased in SG adenoma and SG carcinoma cells, reflecting a maturation-linked expression pattern. Furthermore, in SZ95 sebocytes, naturally occurring lipids including arachidonic acid and arachidonic acid keto-metabolites (e.g. 5-KETE, 12-KETE) appear to regulate PPARγ signaling pathways, which in turn modulate phospholipid biosynthesis and induce neutral lipid synthesis. Collectively, our findings highlight the importance of endogenous ligand-activated PPARγ signaling in human sebocyte biology and suggest that PPARγ might be a promising candidate for the clinical management of SG disorders.

INTRODUCTION

Investigating the function of the sebaceous gland (SG) under normal and pathological conditions is a major research interest in dermatology. This includes investigations into the transcriptional mechanisms that regulate lipid metabolism and inflammatory responses of sebocytes (Toth, et al., 2011). 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. A particularly relevant area of research is the regulation of gene expression by transcription factors responding to changes in fatty molecules and therefore linking metabolic processes to gene regulation.

Peroxisome proliferator-activated receptors (PPARs) are lipid activated nuclear receptors, playing roles in sebaceous lipogenesis, and differentiation (Alestas, et al., 2006; Trivedi, et al., 2006). In particular, the lipid activated nuclear receptor, peroxisome proliferator-activated receptor gamma (PPARγ) plays key roles in adipocyte, liver, macrophage and dendritic cell differentiation as well as in lipid metabolism (Forman et al., 1995; Tontonoz et al., 1998; Kim et al., 2001 Ferre, 2004; Knouff and Auwerx, 2004; Szatmari et al., 2004, Nagy et al; 2012). In addition, PPARγ is expressed in SGs (Deplewski and Rosenfield, 2000; Kim et al., 2001; Zouboulis et al., 2005; Alestas et al., 2006; Trivedi, et al., 2006; Michalik and Wahli, 2007). Furthermore, PPARγ may be an important molecule in acne vulgaris, the most frequent SG-related dermatosis with abnormal lipid storage and inflammation (Anttila et al., 1992; Zouboulis, 2003; Zouboulis et al., 2005; Alestas et al., 2006; Trivedi et al., 2006). Arachidonic acid (AA) markedly enhances lipid synthesis in SZ95 sebocytes (Wrobel et al., 2003; Alestas et al., 2006; Tóth et al., 2009; Géczy et al., 2012) and some of this induction might be regulated via PPARγ (Alestas et al., 2006).

Furthermore, AA is metabolized via various pathways to a large variety of derivates (Harizi et al., 2008), and selected metabolites act as potent endogenous ligands of PPARs, especially of PPARγ in fibroblasts and adipocytes (Forman et al., 1995; Kliewer et al., 1997). However, the nature and identity of endogenous ligands that activate PPARγ 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. (Trivedi, et al., 2006). Previous studies have shown that AA can activate PPARγ; however, it is highly likely that not AA, but some of its metabolites are bioactive mediators of PPARγ activation (Alestas et al. 2006; Thuillier et al., 2002; Kliewer et al., 1995). These findings prompted us to systematically assess the role of PPARγ in human sebocytes. We also sought to identify endogenous PPARγ 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 in normal and pathological SGs and in vitro in a model cell line.

To address these issues, including the expression levels of PPARs and the presence of PPARγ 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 PPARγ expression pattern in sebaceous hyperplasia, adenoma and carcinoma tissues. We found that AA and AA-derived keto-metabolites apparently modulate PPARγ activity, which appears to be important in the regulation of SG differentiation and lipid biosynthesis.

RESULTS

PPARγ and its target genes are expressed in normal human SGs in situ

First, we determined the expression level of PPARγ and markers of its activity in human SGs in situ. Immuno-labeling identified PPARγ protein expression predominantly in the nuclei of normal SGs of skin biopsies obtained from healthy volunteers (n=10) (Figure 1a). 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 PPARγ were examined by quantitative real-time PCR (qPCR). The expression of PPARγ mRNA was higher in healthy SG than in pathologic (hyperplasia and adenoma) samples, although the latter was available only in limited numbers and therefore no definitive conclusions can be drawn (Suppl. Figure 1a).

It is generally accepted that PPARγ activity is indicated by the mRNA expression of its target genes including the adipose differentiation related protein (ADRP) and PPARγ angioprotein related protein (PGAR), as established in other cell types (Forman *et al.*, 1995; Szanto *et al.*, 2004; Nagy and Szanto, 2005; Szatmari *et al.*, 2006; Varga and Nagy, 2008). We also found that laser-microdissected SG samples from healthy individuals express ADRP and PGAR mRNA at high levels (Suppl. Figure 1a). These data suggest that PPARγ and also its target genes are present in human SGs.

PPARγ expression correlates with the differentiation stage of sebocytes

Next we sought to define the role of PPARγ in the process of sebocyte differentiation. Therefore, we investigated SGs from human biopsies obtained from patients with benign or malignant SG lesions. In order to document the differentiation stages of sebocytes within SG, we immunostained sections and measured mRNA for Ki67, a well known marker of active cell cycle, and for the androgen receptor (AR). Also, the grade of differentiation of sebocytes within the SG was classified. Immunostaining was scored semi-quantitatively according to previous reports (Cottle *et al.*,2013, Tsujita-Kyutoku et al. 2003) (Figure 1b and Suppl. table 1.). In order to extend these analyses we used reference markers for basal immature sebocytes p63 protein (Tsujita-Kyutoku *et al*, 2003). PPARγ was present at high levels in sebocytes of all samples (n=10) from patients with SG hyperplasia (Figure 1a). Both the Ki67 and p63 markers stain nuclei of immature sebocytes located at the basal layers of normal SG, whereas AR was incorporating predominantly the basal and suprabasal cell layers, and – to a lesser extent the central cells, as well. By contrast, PPARγ was detectable in terminally differentiated mature sebocytes located in the central regions of normal SGs, but was hardly detectable in basal layer cells. Double immunolabeling for Ki67 along with PPARγ clearly demonstrated the predominantly different localizations for these two nuclear proteins discriminating the immature (Ki67 positive) cells from the mature (PPARγ positive) ones, which appeared to be the rule for these marker expressions in normal and hyperplastic SGs. We also analyzed samples of SG-tumours. When compared to normal and hyperplastic SGs, cells of sebaceous adenomas (n=10) generally expressed decreased amounts of PPARγ, which appeared to decrease further in malignant tissues and sebaceous carcinomas (n=9) and down to hardly detectable levels in poorly differentiated neoplastic cells. In the same SG carcinoma samples, the un-affected normal tissue parts with intact sebaceous glands retained the significantly high PPARγ-specific nuclear protein expression levels (Suppl. Fig 1c). The decreased level or the absence of PPARγ protein correlated with an increase of Ki 67 and p63 positive neoplastic cells of sebaceous carcinoma cases, reflecting the ratio of more differentiated (i.e., PPARγ-positive cells with lipid droplets) and the less mature (poorly differentiated, basaloid) cellular components of the tumors in all the cases studied. Double immunostaining with Ki67 and PPARγ, confirmed that the more differentiated carcinoma cells expressed preferentially PPARγ without Ki67, and the Ki67 positive proliferating pleomorphic neoplastic cells did not exhibit PPARγ (Figure 1, last image at lower right). In contrast, the expression pattern of AR at protein levels generally did not show such an obvious and clear distinction in well differentiated and poorly differentiated cellular

components of sebaceous carcinomas studied so far. However it was expressed in high proportion of adenoma cells in all cases (Figure 1, middle).

We then corroborated our results by investigating the PPARγ 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; from the facial region of 5 different donors. Subsequently, mRNA expressions of PPARγ, ADRP and PGAR, Ki67, AR were measured (Figure 1d). PPARγ, ADRP and PGAR were expressed at low levels in basal cells, whereas higher expression was detected in the well-differentiated sebocytes of the central zone 4/5 samples (PPARγ), 3/5 samples (ADRP, PGAR). In contrast Ki67 and AR mRNA was expressed at high levels in the basal cell layer, and was expressed to a lesser degree or not detectable in central cells (AR 4/5 samples, Ki67,5/5 samples). These data together with findings from the 10 samples analyzed by immunohistochemisty indicated that PPARγ may be used as a potential marker of sebocyte differentiation.

Active PPARγ is present in human SZ95 sebocytes

To further explore how PPARγ functions in sebocytes we used a well-established human SG cell line, SZ95 sebocytes (Zouboulis *et al.*, 1999). Expressions of the three PPAR isoforms were detected at the mRNA level in SZ95 sebocytes (Suppl. Figure 1b). Remarkably, PPARγ mRNA expression was highest among the PPAR isoforms. We measured the mRNA levels of RXR isoforms, which are the heterodimerization partners of PPARγ. Confirming a previous report (Tsukada et al., 2000) RXRα appeared to be the dominant RXR subtype in SZ95 sebocytes (Suppl. Figure 1b).

Next, we investigated PPARγ activity in SZ95 sebocytes. We treated SZ95 sebocytes with the synthetic PPARγ agonist rosiglitazone (RSG) and measured the mRNA levels of two target genes, PGAR and ADRP. RSG (1-10 μM) induced significant upregulation of PGAR (ca. 32-fold) and ADRP (ca. 3-fold). The RXR agonist LG100268 (20-200 nM) also increased PGAR (ca. 46-fold) and ADRP (ca. 2-fold) expressions, respectively (Suppl. Figure 1b). Administration of a PPARγ inhibitor, GW9662 markedly abrogated the RSG-induced target gene induction in a dose-dependent manner (1-10 μM). These data collectively suggest that PPARγ is active in human SZ95 sebocytes. This confirms our previous observations

(Dobrosi *et al.*, 2008). Our findings also suggest that PPARγ and RXRα are likely to act on the same molecular pathways .

AA regulates sebocyte differentiation, in part via PPARγ-coupled signaling pathways

To further explore the biological consequences of PPARγ stimulation on SG biology, we also investigated the functional role of PPARγ in SZ95 sebocytes. Sebocyte differentiation is characterized by the synthesis of various lipids, among which neutral lipids represent the major part (Zouboulis *et al.*, 1999; Rosenfield *et al.*, 1998). Confirming our previous results (Toth *et al*, 2009; Alestas *et al.*, 2006), first we showed that AA, one of the most effective differentiation inducer of SZ95 sebocytes, markedly enhanced lipid synthesis of the cells (Figure 2a). Quantitative Nile red-based fluorescence assay was performed, which showed that AA induced neutral lipogenesis in SZ95 sebocytes in a dose-dependent manner (Figure 2c.).

Intriguingly, the PPARγ agonist RSG also induced sebaceous neutral lipid synthesis in SZ95 sebocytes (albeit at a lower potency than AA) (Figure 2 a and c). We then assessed the involvement of PPARγ in AA induced biological changes. AA treatment of the cells resulted in a marked upregulation of the mRNA expression levels of the PPARγ target genes (PGAR and ADRP) which effect could be fully abrogated by a PPARγ specific antagonist GW9662 (Figure 2d-e). Moreover, the lipid synthesis-promoting activity of AA was also inhibited partially by a PPARγ antagonist. Finally, we found that AA treatment also upregulated the mRNA level of PPARγ (Figure 2b). In order to determine the specificity of AA treatment, we also treated the sebocytes with oleic acid (OA) and palmitoleic acid (PA), interestingly both induced lipid accumulation and PGAR and ADRP expression, but neither effect was inhibited by PPARγ antagonist treatment (Figure 2d-e) suggesting that PPARγ is not involved in these responses.

AA regulates the production of selected lipid molecules via PPARγ

To further explore how PPARγ activation affects lipid production in sebocytes, we treated the cells with AA (25 μM) and with a PPARγ antagonist GW9662 (10 μM) 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) (Figure 3a) 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.

Densitometric analyses of HPTLC spots showed that TG levels were significantly elevated by AA and this induction was inhibited by a PPARγ inhibitor (Figure 3a). 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 (Figure 3b). Biosynthesis of TG is a multistep process that involves different lipid intermediates. To gain insight into the mechanisms involving PPARγ 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 (Figure 3c). When AA was co-administered with a PPARγ antagonist this induction of TG 60:12 was inhibited, which is consistently with the HPTLC results concerning the total TG (Figure 3a-c).

The other neutral lipids that were influenced by the addition of AA were CE and WE. AA elevated WE levels, however no effect of the PPARγ antagonist on this was observed. Additionally, AA had no significant effect on the total CE levels regardless of the presence of the PPARγ antagonist (densitometric results not reported). Interestingly, basal CH level was significantly decreased by the PPARγ antagonist GW9662 as demonstrated by the quantitative analysis of CH by HPLC-MS (Figure 3b). In contrast, concentration of SQ determined by HPLC-MS was not affected by AA alone or in combination with the PPARγ antagonist GW9662 (data not shown).

Next, using direct flow injection electrospray ionization tandem mass spectrometry (ESI-MS/MS), ten main specific membrane lipid groups were assessed: phosphatidylinositol, lysophosphatidylcholine, phosphatidylcholine, sphingomyelin, dihydrosphingomyelin, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, ceramide and glucosylceramide. AA had no significant effect on ceramides and glucosylceramides. Among the other 8 measured membrane lipid groups, AA induced significant alterations in the levels of 136 of the 198 measured lipid species (Figure 3d). Importantly, the presence of the PPARγ inhibitor GW9662 significantly prevented the action of AA on 8 lipid species (Figure 3d). 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 PPARγ dependent fashion.

Therefore, the AA-coupled PPARγ 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 PPARγ activation.

The above data collectively suggest that i) PPARγ is indeed active in cultured SZ95 sebocytes where its activation resulted in a lipogenic action; ii) AA acts as a PPARγ agonist in SZ95 sebocytes; and iii) the effect of AA to enhance lipogenesis and hence sebaceous differentiation was only partially mediated by the activation of PPARγ and the related signaling (and hence other, PPARγ-independent signal transduction mechanisms are most likely involved) iiii) PGAR and ADRP can be regulated by other lipid mediators, and AA's effect is only partly PPARγ dependent. As an additional control we measured cell line viability with MTT, and none of the applied ligands induced cell death (Suppl. Figure 1b).

AA metabolites activate PPARγ and induce lipogenesis in sebocytes

AA can be metabolized to a plethora of signal lipids (Harizi *et al.*, 2008). 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. Among the several candidates, we primarily focused on eicosanoids which have a proven ability to activate PPARγ like 5-KETE, 12-KETE and 15-KETE.

Upon AA treatment, we detected increased concentrations of 5-KETE, 12-KETE, and 15-KETE (Figure 4a). Furthermore, these endogenous AA metabolites administered at a concentration of 5 μM (Shiraki *et al.*, 2005; O'Flaherty *et al.*, 2005) significantly elevated sebaceous lipid synthesis (Figure 4b-c), and in case of 5-KETE, 12-KETE, neutral lipid production was partially inhibited by PPARγ antagonist GW9662. To explore whether these molecules also activate PPARγ in another set of experiments, SZ95 sebocytes were treated similarly and expressions of PPARγ target genes were then assessed. We found that 5-KETE, 12-KETE and 15-KETE significantly increased the gene expression levels of PGAR and ADRP. However, the PPARγ antagonist GW9662 was able to inhibit the induction of PGAR only in the case of 12-KETE and 5-KETE (Figure 4d), and the induction of ADRP in the case of 12-KETE (Figure 4e). 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 PPARγ.

DISCUSSION

The fact that PPARγ is expressed in normal SG, in primary sebocytes and in SZ95 sebocytes has been well documented (Smith and Thiboutot, 2008). However, it was not know what might be the role of this transcription factor in situ. We intended to extend these studies by the combination of using laser microdissection and cellular models. We found that PPARγ appears to correlate with the more differentiated state of sebocytes. This was confirmed by using established markers of cell cycle activity and differentiation such as Ki67, p63 and AR molecules. Co-staining of Ki67 and PPARγ molecules gave indication of an inverse relationship. AR was expressed mostly in basal and parabasal cell layers, and showed an overlap with PPARγ expression in the parabasal layer. We also compared the expression of these molecules in pathologies. In sebaceous hyperplasia and found that the expressions of PPARγand the other markers were essentially the same as in normal SG. In contrast, the proportion of cells expressing PPARγ in sebaceous adenoma and sebaceous carcinoma is lower, but the proportion of Ki67, and p63 positive cells is higher (Figure 1a). Recently,Cottle at al. described the expression of AR (basal, parabasal layer), Ki67 (some is basal layer) and PPARγ (intermediate, i.e. outer central layer)in healthy SG of murine origin and gatheredsimilar findings with AR in SG tumours. Additionally, the differentiation stage of carcinomas appeared to be correlated with the presence of PPARγ in tumour cells (Suppl. Figure 1c). These findings are consistent with the results obtained using uroepithelial carcinoma cells, in which the presence of PPARγ changed consistently with the differentiation state of carcinoma cells (Nakashiro *et al.*, 2001) and also (estrogen/progesterone) receptor expression in breast cancers (Payne *et al.*, 2008). This suggests that PPARγ might be a reliable diagnostic marker of sebocyte differentiation state.

As far as the function of the receptor is concerned we had to rely on a model cell line. Using a model of AA-treated differentiated sebocytes (Alestas *et al.*, 2006; Wrobel *et al.*, 2003), we observed that the lipid rich SZ95 sebocytes expressed high levels of PPARγ and also its target genes PGAR and ADRP (Suppl. Figure 1b), consistent with our previous finding, where the AEA (endocannabinoid) induced differentiation/lipid production was shown to be induced via PPARγ (Dobrosi *et al*, 2008). In addition, we provide pharmacological evidence indicating that the specific PPARγ antagonist GW9662 dose - dependently inhibited this AA mediated target gene induction (Suppl. Figure 1b). Also, the RXR agonist LG100268 induced ADRP and PGAR mRNA elevation. Our interpretation of these data is that both PPARγ and

RXRα are active in human SZ95 sebocytes, and possibly are heterodimer partners, or act on the same molecular pathways (Suppl. Figure 1b).

Prior research has shown that RSG-activated PPARγ has a role in sebaceous lipid production, a marker of sebocyte differentiation (Trivedi *et al.*, 2006). In our study both the natural ligand precursor, AA, and the synthetic ligand, RSG, induced sebaceous lipogenesis (Figure 2b) similar to previous results (Trivedi *et al.*, 2006). This suggests that PPARγ-induced gene activation coincides with sebocytes' neutral lipid production. We analyzed neutral lipids, which are the major components of the sebaceous lipid mixture, along with membrane lipid species (Figure 3b). We found that, TG levels were significantly elevated by AA, and this effect was inhibited by PPARγ antagonist. It was previously shown, that PPARγ ligand RSG induced TG production (Trivedi et al, 2006). These findings indicate that the elevation of TG by AA was the result at least partly of PPARγ activation, and AA takes part in TG formation as demonstrated by the elevation of AA-containing species among the total TG.

It is known that in sebocytes, RSG also induced cholesterol accumulation (Trivedi et al, 2006). In our data, inhibition of PPARγ 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 remodelling 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 PPARγ in sebocytes, It is also likely that pathways leading to neutral lipid formation were regulated by AA in a lipid species selective manner.

AA treatment also causes PPARγ dependent elevation of some minor lipids, like LPC and PIs (Figure 3c). In sebocytes, LPC is a minor phospholipid in membranes of lipid droplets consisting of monolayer phospholipids (Corsini et al., 2003). 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 (Brasaemle *et al*, 1997; Heid *et al.*, 1998), and were shown to have ADRP protein on their surface. These data might indicate that PPARγ 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 PPARγ-dependent manner suggesting that enzymes, which are involved in the synthesis/degradation of PIs are also influenced by the receptor. The altered level of PIs, based on their versatile roles in various biological processes, might influence several functions of sebocytes, i.e. PIs are essential phospholipids in the formation of cellular membranes. Moreover, one group of their metabolic products, 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 PPARγ also plays a role (Dobrosi *et al*., 2008).

Phopholipase A_2 (PLA2) isthe enzyme type responsible for hydrolyzing fatty acids like AA from glycerophospholipids (Scott, 1997). Therefore they represent the source for the AA production. PLA2 was detected in various cells of skin and also in SZ95 sebocytes (Schagen*et al.* 2008) and treatment with active PLA2 increased neutral and polar lipid production of sebocytes (Schagen*et al.* 2008). It seems possible, that PLA2 is one regulator of endogenous AA production in sebocytes, and it also determines the level of PPARγ activation in these cells at least in part. The identification of endogenous AA metabolites (such as 5-KETE, 12-KETE) by us 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 is exogenously added.

Although PPARγ probably controls both the levels of major neutral lipids and levels 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 endogenous ligands. Various AA metabolites have been described as natural PPARγ ligands in the skin (Weindl *et al.*, 2005), adipocytes (Oster, *et al.* 2010), cardiomyocytes (Hovsepian *et al.*, 2010) and in several types of cancers (Maggiora, *et al.*, 2010 ; Li *et al.*, 2010; Dionne *et al.*, 2010). We found that the AA keto-metabolites (5-KETE and 12-KETE) act as endogenous PPARγ ligands in SZ95 sebocytes as they elevated sebaceous lipid synthesis in a PPARγ-dependent manner (Figure 4b-e).

In summary, our study provides evidence that PPARγ participates in the signaling mechanisms of sebocyte differentiation. We also demonstrated that AA-activated PPARγ is involved in the regulation of major neutral lipid and phospholipid biosynthesis. Finally, we presented that PPARγ is activated by AA keto-metabolites (5-KETE, 12-KETE). Taken together, our data implicate that AA activated PPARγ is an important regulator of differentiation and lipid metabolism in human sebocytes; therefore, PPARγ might be a potential therapeutic target molecule in sebaceous dermatoses.

MATERIALS AND METHODS

Detailed description of the employed techniques can be found in Supplementary data.

Cell culturing

Human immortalized sebocytes were cultured according to previously described protocol. (Zouboulis *et al.*, 1999).

Determination of intracellular lipids

For quantitative measurement of lipid content, SZ95 sebocytes (10,000 cells/well) were cultured in 96-well black-well/clear-bottom plates (Greiner Bio-One, Frickenhausen, Germany) in quadruplicates and were treated with compounds for 24 hrs. Then, cells were stained with 1 μg/ml Nile red (Sigma-Aldrich), and incubated at 37°C for 20 min. Fluorescence was measured on a Molecular Devices FlexStation^{384} II Fluorescence Image Microplate Reader (FLIPR, Molecular Devices, San Francisco, CA USA). Results were expressed as percentages of the relative fluorescence units (RFU) in comparison with the controls using 485 nm excitation and 565 nm emission wavelengths for neutral lipids, and 540 nm excitation and 620 nm emission wavelength for polar lipids (Alestas et al., 2006; Toth *et al*., 2009). Mean ± SD of quadruplicate determinations is shown. Experiments were repeated 3 times.

Determination of lipid species by ESI-MS/MS, HPTLC, and HPLC-MS

The composition of neutral lipids was investigated in SZ95 sebocytes based on the HPTLC and HPLC -MS with a time of flight MS detector (HPLC-ToF/MS) analyses of WE, TG, DG, CE, CH, FFA, and SQ (Fuchs *et al*. 2011, Camera *et al*. 2010). Quantitative determinations of AA, SQ and CH were performed with a triple quadrupole mass detector (QqQ) following HPLC separation (Camera et al., 2010). The HPLC-MS systems were from Agilent Technologies (Waldbronn, Germany). For quantitative measurement of 9 membrane lipid classes in SZ95 sebocytes (PI, LPC, SPM, dhSPM, PE, PS, PG, CER, gluCER), direct flow injection electrospray ionization tandem mass spectrometry (ESI-MS/MS) in positive ion mode was applied, using the analytical setup and strategy described previously (Liebisch *et al*. 2002, Liebisch *et al.* 2004; Liebisch *et al.* 2006).

Detemination of eicosanods using HPLC-MS-MS methodology

For quantitative determination of eicosanoids and docosanoids in treated SZ95 cells, the HPLC-MS-MS methodology was used; the analytical sample preparation procedure is based on an established method used for retinoid quantification (Rühl *et al*., 2006) and was recently published for organs and serum analysis (Szklenar *et al*., 2013 in press).

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,using Superscript II reverse transcriptase (Invitrogen). Quantitative PCR was performed on an ABI PRISM 7900 instrument (Applied Biosystems Foster City, CA, USA), 40 cycles of 95° C for 12s and 60°C for 1 min using TaqMan assays (Applied Biosystems). All PCR reactions were performed in triplicates with one control containing no RT enzyme. The comparative Ct method was used to quantify transcripts and normalize for human cyclophilin A. Mean and SD were calculated for both the normalized and the normalizer values for all experiments. Data shown are mean±SD of three biological replicates measured in triplicates. * $P< 0.05$ was considered as significant change compared to vehicle treated control, or ligand treated cells.

Statistical analysis

When applicable, data were analyzed using a two tailed un-paired t-test (Figure 1.d, Figure 4 a) and values of $P<0.05$ were regarded as significant. In addition statistical differences were further verified using 1-way ANOVA with Bonferroni and Dunnett post hoc probes, resulting in similar results (Figure 2 b-e, Figure 3 a-d, Figure 4 b-e, Suppl. Figure 1b).

Patient samples

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

Immunohistochemistry (IHC) and double immunofluorescence (IF)

IHC for PPARγ was carried out using the biotin-free and tyramid-based CSAII kit (Dako, Glostrup, Denmark) according to the manufacturer's instruction and as described previously (Szanto *et a.l,* 2004; Szatmari *et al*., 2006; Gogolak *et a.l,* 2007). Single IHC staining for androgen receptor (AR), and p63, and Ki67 were all detected with standard immune-peroxidase method using an EnVision^{+}-HRP (horse-raddish-peroxidase) detection kit or an automated immune-stainer (Bond-Leica, UK) utilizing VIP (purple) or DAB (brown) chromogenic substrates for microscopic visualisation. The immunostained slides were then

evaluated semi-quantitatively for distinct sebaceous glands/SG tumors reflecting the % of positive cells: -, negative; ***1***+, 0-20%; ***2***+, 20-50%; ***3***+, 50-75%; ***4***+, 75-100%.

Laser Capture Microdissection and RNA preparation

Serial cryosections were then made and mounted onto sterile membrane-containing microscopic slides (Jenkins *et al*., 2005). Compartments of SGs were in situ laser-microdissected (MMI-Olympus LCM system, Glattbrugg, Switzerland) and collected separately from the surrounding dermal tissue (Suppl.Fig. 1, c).Then, Absolutely Nanoprep RNA isolation kit (Stratagene) was used according to the vendor's protocol. Samples were stored on -70ºC until use.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

Figure 1. Comparative analyses of protein and mRNA expression patterns of PPARγ in conjunction with compartment-associated reference markers for human normal and pathological sebaceous glands (SG) and laser-microdissected healthy SG compartments.

a, HE-stained histology and the corresponding immunoprofiles in human skin tissues for PPARγ, p63, AR and Ki67 nucleo-protein expressions of various sebaceous gland (SG) types, respectively. Black arrows indicate representative positive cells (brown or purple nuclei). Note the comparable expression patterns of PPARγ (2nd column) and the reference

markers p63 and Ki67 (3rd and 5th column) in normal and hyperplastic SGs demonstrating clear discriminations for the PPARγ^{+} differentiated sebocytes with vacuolated (lipid rich) cytoplasm from the thin layers of p63/Ki67^{+} immature basal sebocytes exhibiting inconspicuous cytoplasm. As opposed, the sebaceous adenoma samples (3rd-line panel) show an increase number of p63/Ki67^{+} basal immature (proliferating) sebocytes, on the account of differentiated sebocytes as the latter cell types show a relative reduction in number with a decreased PPARγ expression intensities - albeit their morphologies still exhibit lipid droplets with no pleomorphism reflecting terminal maturation. As compared to adenoma, sebaceous carcinomas show prominent decrease in the number and expression intensities of PPARγ^{+} neoplastic malignant cells in the function of differentiation levels (i.e., grade of pleomorphism) of lesional sebocytes. Accordingly, poor differentiation is indicated by cellular pleomorphism and significantly increased Ki67^{+} cells with no PPARγ expression, while in well or moderately differentiated regions (within the same tumor) PPARγ^{+} cells can be identified. This phenomenon is clearly demonstrated on the PPARγ-Ki67 double-labeled images by means of both immuno-peroxidase and immunofluorescence methods (lower right images). **b**, Summary of immunostaining and differetiation grade of sebocytes in the lesion. Samples were evaluated semi-quantitatively for distinct sebaceous glands /SG tumors, reflecting the % of positive cells: -, negative; *1*+, 0-20%; *2*+, 20-50%; *3*+, 50-75%; *4*+, 75-100%. **c,** Basal and subrabasal (white column) and central (black column) cell layers of normal SG samples were laser microdissected (n=5 samples from 5 donors, ie.S6:sample6), for mRNA analysis. Scale bar 100μm. **d,** PPARγ, ADRP, PGAR, AR and Ki 67 expression at the mRNA level. The values shown are mean ±SD of three separate measurements. * P< 0.05 was considered as significant change.

Figure 2. AA induces upregulation of PPARγ and PPARγ target genes resulting in differentiation of SZ95 sebocytes

(**a-c**) SZ95 sebocytes were treated for 24h or (**d-e**) 6h with 1-10 μM RSG, 1-10 μM GW9662 (PPARγ inhibitor), 5-50 μM AA, 5-100 μM OA, 5-100 μM PA and combinations of these ligands. **a)** Intracellular lipids were assessed by Nile red labeling following fluorescence microscopy. Scale bar: 50 μm **b)** Quantitative measurement of neutral and polar lipids by FLIPR fluorescent microplate reader. Data (means ± SD) are presented as percentage values

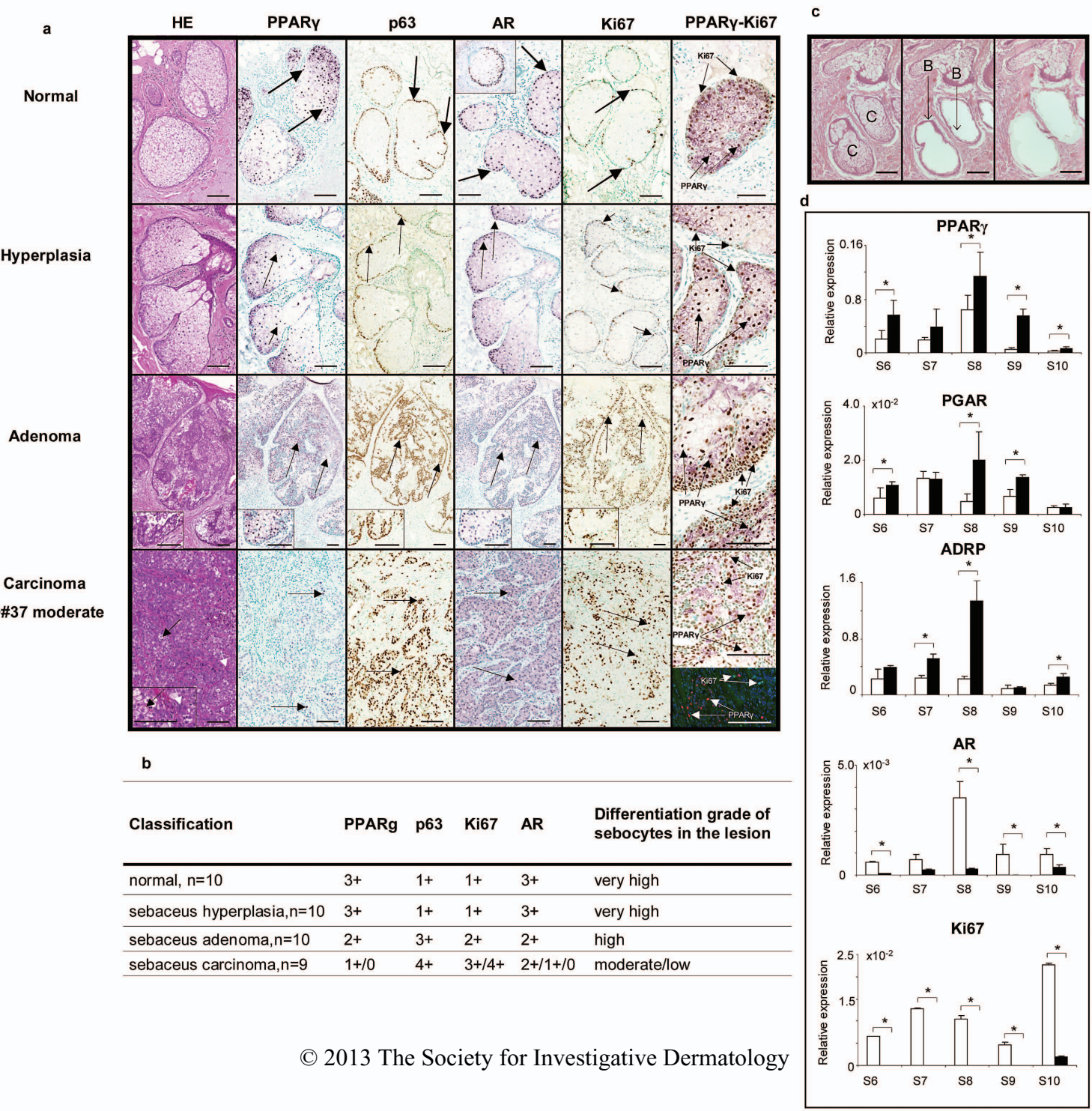
of quadruplicates; the value of the vehicle treated control group was defined as 100%. Two additional experiments yielded similar results. **c-e)** mRNA expression of PPARγ, PGAR, and ADRP. AA increased the mRNA expression of PGAR and ADRP in a dose-dependent manner which was inhibited by GW9662. Mean±SD of three biological replicates measured in triplicates. * P< 0.05 was considered as significant change compared to vehicle treated control, or ligand treated cells.

Figure 3: Arachidonic acid induced PPARγ activation changes selected lipid classes in SZ95 sebocytes. Cells were treated for 24 h with 25 μM AA or AA 25 μM plus 10 μM GW9662. **a,** HPTLC and densitometric analysis of TG bands (average ± SD of normalized intensity); **b**, Quantitative determinations of AA and CH by HPLC-MS with triple quadrupole (QqQ) analyzer (average ± SD of normalized concentrations); **c,** HPLC-MS analyses with a time of flight MS detector (ToF) of AA containing DG 40:8 (20:4/20:4) and TG 60:12 (20:4/20:4/20:4). AA elevated the levels of DG and TG, but only TG 60:12 induction was significantly inhibited by the PPARγ antagonist GW9662; **d,** ESI-MS/MS analysis of 10 membrane lipid classes: phosphatidylinositol, lysophosphatidylcholine, phosphatidylcholine, sphingomyelin, dihydrosphingomyelin, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, ceramide and glucosylceramide. AA changed the level of 136 lipid types significantly. Heat map of ESI-MS/MS of PI (phosphatidyl inositol) and LPC (lysophosphatidylcholine) 8 lipid species, where AA effect was inhibited by PPARγ antagonist GW9662. Color bar defines concentration fold change. Different fatty acid chain length and saturation of lipids are shown in numbers. Data are expressed as mean of three independent determinations of three independent biological replicates (P<0.05).

Figure 4. Arachidonic acid-derived metabolites 5-KETE and 12-KETE are potential endogenous lipogenic activators of PPARγ in SZ95 sebocytes

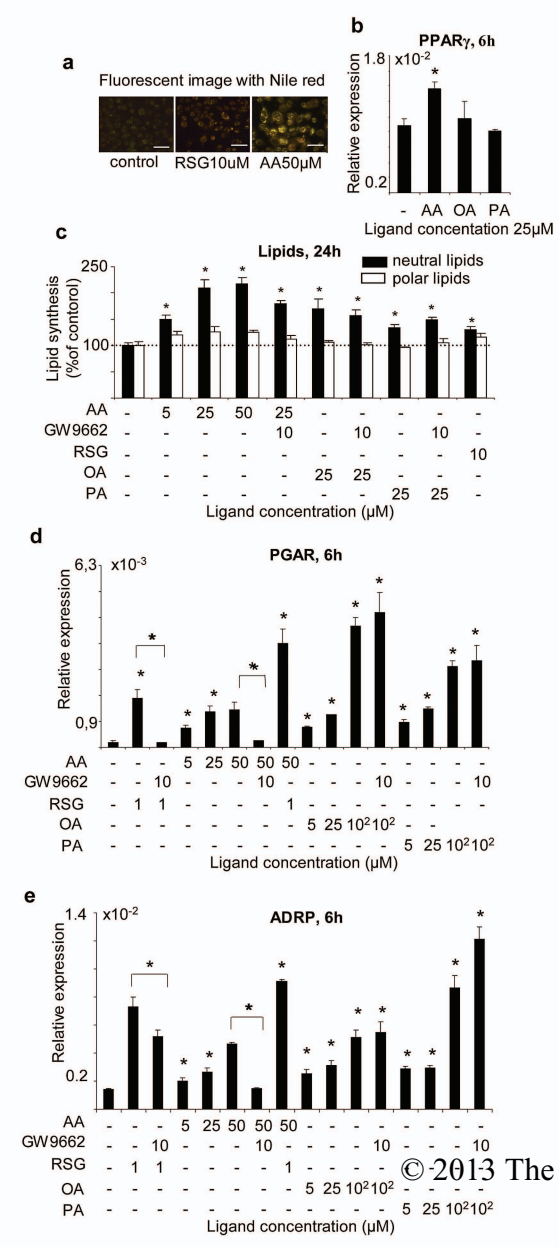
a) SZ95 sebocytes treated for 6 hours with 25 μM AA and then intracellular concentrations of its keto-metabolites (5, KETE, 12-KETE, 15-KETE) were measured with HPLC-MS technology. Heat map of concentrations; color bar defines concentration fold change. **b-e**) SZ95 sebocytes were treated with 10 μM (b-c) or 1uM RSG (d-e), 25 μM AA, 10 μM GW9662, 5 μM 5-KETE, 5 μM 12-KETE, 5μM 15-KETE and combination of these ligands with a PPARγ-antagonist. Quantitative measurement of neutral **(b)**and polar **(c)** lipids by FLIPR fluorescent microplate reader after 24 h treatment. Data (means ± SD) are presented

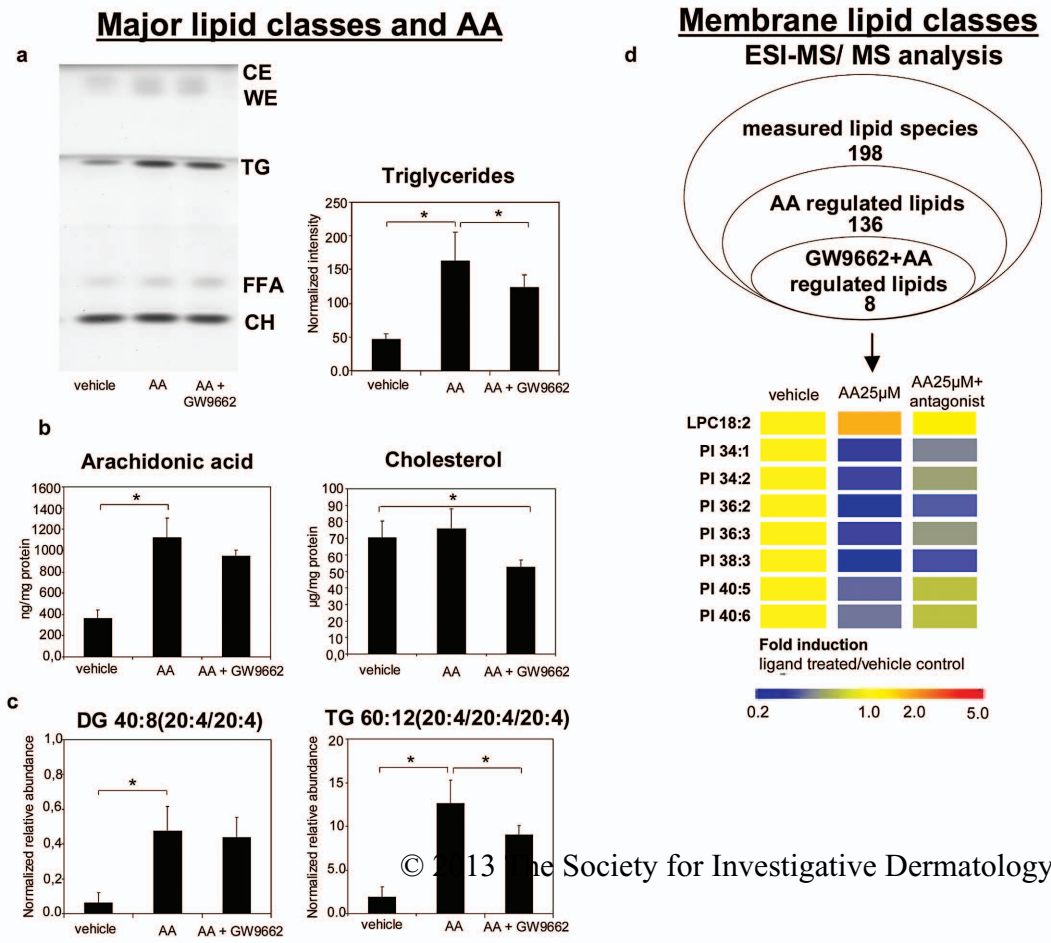
as percentage values; the value of the vehicle treated control group was defined as 100%. Two additional experiments yielded similar results. mRNA expression of PPARγ target genes PGAR (**d)** and ADRP **(e)**. Data are expressed as mean± SD of triplicate determinations of three independent biological replicates ($P<0.05$). $P< 0.05$ was considered as significant change; *compared to vehicle treated control; # compared to the treated group without the presence of GW9662.

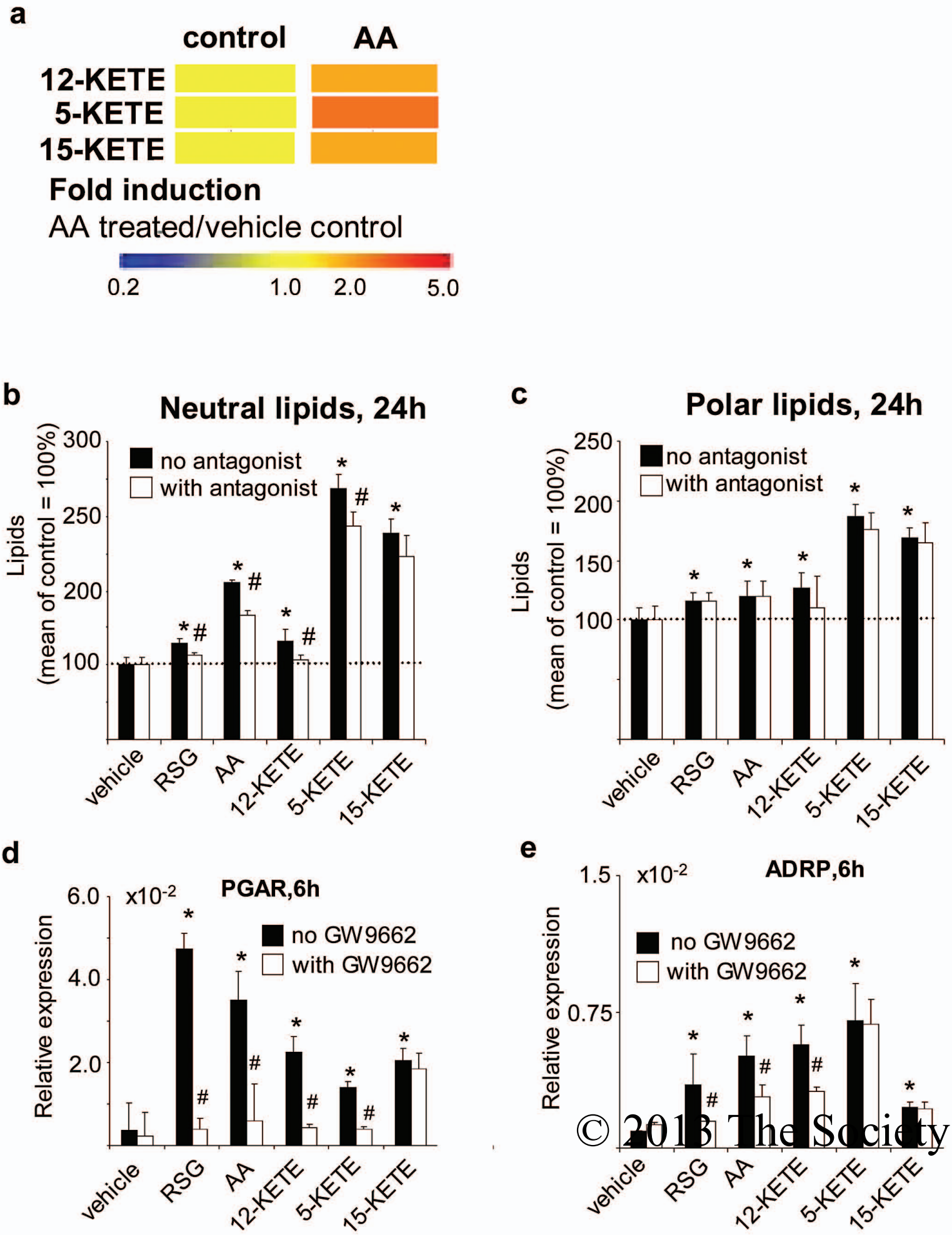


b

Classification	PPARg	p63	Ki67	AR	Differentiation grade of sebocytes in the lesion
normal, n=10	3+	1+	1+	3+	very high
sebaceus hyperplasia,n=10	3+	1+	1+	3+	very high
sebaceus adenoma,n=10	2+	3+	2+	2+	high
sebaceus carcinoma,n=9	1+/0	4+	3+/4+	2+/1+/0	moderate/low


a
Fluorescent image with Nile red
control
RSG10uM
AA50μM
b
PPARγ, 6h
x10-2
1.8
0.2
Relative expression
- AA OA PA
Ligand concentation 25μM
c
Lipids, 24h
neutral lipids
polar lipids
250
100
Lipid synthesis (%of control)
AA - 5 25 50 25 - - - - -
GW9662 - - - - 10 - 10 - 10 -
RSG - - - - - - - - - 10
OA - - - - - 25 25 - - -
PA - - - - - - - 25 25 -
Ligand concentration (μM)
d
PGAR, 6h
x10-3
6,3
0,9
Relative expression
AA - - - 5 25 50 50 50 - - - - - - -
GW9662 - - 10 - - - 10 - - - - 10 - - - 10
RSG - 1 1 - - - - - 1 - - - - - - -
OA - - - - - - - - - 5 25 10² 10² - - -
PA - - - - - - - - - - - - - 5 25 10² 10²
Ligand concentration (μM)
e
ADRP, 6h
x10-2
1.4
0.2
Relative expression
AA - - - 5 25 50 50 50 - - - - - - -
GW9662 - - 10 - - - 10 - - - - 10 - - - 10
RSG - 1 1 - - - - - 1 - - - - - - -
OA - - - - - - - - - 5 25 10² 10² - - -
PA - - - - - - - - - - - - - 5 25 10² 10²
Ligand concentration (μM)




a
control
AA
12-KETE
5-KETE
15-KETE
Fold induction
AA treated/vehicle control
0.2
1.0
2.0
5.0
b
Neutral lipids, 24h
Lipids
(mean of control = 100%)
no antagonist
with antagonist
vehicle
RSG
AA
12-KETE
5-KETE
15-KETE
c
Polar lipids, 24h
d
PGAR,6h
x10-2
Relative expression
no GW9662
with GW9662
e
ADRP,6h
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