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Investigation of novel aspects of the endocannabinoid signaling in human corneal epithelial cells and sebocytes

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In case if one would like to participate on the defense, please indicate it on the e –mail address angyal.agnes@med.unideb.hu by the last working day before the exam (05/07/2022) until 4PM CET.

Introduction

The main research focus of our team is to study various human skin diseases, with a special emphasis on the molecular background of these diseases and on the putative role of the endocannabinoid system (ECS). The ECS is a complex signaling system. Depending on the inclusivity of the definition, it may include dozens of endogenous ligands (e.g., anandamide [AEA]), several metabotropic (e.g. CB_1, CB_2, GPR55, GPR119, etc.), ionotropic (various transient receptor potential [TRP] ion channels) and intranuclear (peroxisome proliferator-activated receptors [PPARs]) receptors, as well as enzymes involved in the synthesis (e.g., N-acylphosphatidylethanolamine-specific phospholipase D [NAPE-PLD], diacylglycerol lipase [DAGL]-α and -β), degradation (e.g., fatty acid amide hydrolase [FAAH], monoacylglycerol lipase [MAGL]) and transmembrane transport (the putative endocannabinoid [eCB] membrane transporter [EMT]) of the ligands.

Our group has previously shown that locally produced eCBs, such as AEA and 2-arachidonoylglycerol (2-AG), enhance the synthesis of sebaceous lipids by activating the CB_2 receptor → extracellular signal-regulated kinase (ERK)-1/2 mitogen-activated protein kinase (MAPK) → peroxisome proliferator-activated receptor (PPAR)-γ pathway. We also demonstrated that human sebocytes express key enzymes involved in the synthesis and degradation of eCBs at both mRNA and protein levels, and pharmacological inhibition of the EMT moderately enhanced sebaceous lipid production and resulted in anti-inflammatory effects, most likely via increasing the eCB tone. Furthermore, our experiments led to the unexpected finding that, in addition to AEA and 2-AG, sebocytes are involved in the metabolism of the "non-classical" eCB oleoyl ethanolamide (OEA).

Because by the time of our experiments, no data were available on the expression and possible role of OEA and its main receptor (GPR119) in human sebaceous glands, in the first half of our experiments, we have investigated the biological effects of OEA in human sebocytes. We were especially interested in studying whether these molecules may play a role in the inflammatory processes as well as the pathological elevation of the production of sebaceous lipids and in acne.

In the second half of our experiments, we examined the ECS of another important barrier, namely the cornea. The cornea is the anterior, translucent part of the outer protective covering of the eyeball. Its role is to protect the structures inside the eye, and it also plays a key role in the refraction of light that enters the eye. The corneal epithelial cells form the outermost histological layer of the cornea and they are the first cells to encounter stimuli, physico-chemical agents and microbes from the outside world. By creating a physical and immunological barrier, these cells are not only passive sufferers of this encounter, but also respond to them adequately, e.g., by producing cytokines and chemokines that influence local inflammatory processes. Damage to the corneal epithelium can trigger inflammation, which can lead to the impairment of the barrier or, if the damage is more extensive, even to the vascularization of the cornea resulting in the loss of vision.

TRPV1, one of the TRP channels mentioned above, plays an important role in the development of the corneal inflammatory response, and its activation can exacerbate the inflammatory processes in the cornea. Importantly, CB_1 is also expressed on human corneal epithelial cells, and its activation by AEA could prevent the inflammatory effect of TRPV1 activation. Given that literature data suggest that AEA (together with other eCBs) is present in human corneal epithelial cells, these results suggest that the AEA-dependent CB_1 activation may establish a continuous anti-inflammatory tone in these cells.

Since, under physiological conditions, the cornea is an avascular tissue, the eCBs detected here could not have been derived directly from the circulation, suggesting that some corneal cells may be capable of *de novo* synthesis of eCBs, but, at the time of our experiments, no data were available in the literature on the expression of the enzymes involved in the metabolism of eCBs. Thus, in our experiments involving corneal epithelium, our aim was to investigate the expression of the main enzymes of eCB metabolism (see above) in the cornea and to study the potential anti-inflammatory effect of AEA in clinically relevant inflammatory conditions. To achieve the latter aim, we used UVB irradiation (to model photodamage) and Toll-like receptor (TLR)-3 activator polyinosinic-polycytidylic acid (p(I:C)) treatment (to model viral keratitis). Considering that primary human corneal epithelial cells are essentially impossible to obtain, our experiments were performed using a recombinant SV40-adenovirus vector in an immortalized human corneal epithelial cell line (HCEC).

Aims

In light of the above considerations, within the confines of the current study, we aimed to answer the following questions:

1. What is the effect of OEA on the biological processes of human sebocytes and what is the mechanism of action?

2. Which members of the ECS are present on human corneal epithelial cells?

3. What is the effect of AEA treatment on the inflammatory responses of HCECs to UVB irradiation and TLR3 activation?

Materials and Methods

Culturing and treatment of human immortalized SZ95 sebocytes and HCECs

SZ95 sebocytes were cultured in Sebomed™ Basal Medium supplemented with 10% (V/V) heat inactivated fetal bovine serum, 1 mM $CaCl_2$, 5 ng/ml human recombinant epidermal growth factor, and MycoZap™ Plus-CL. The medium was changed every two days. and, in order to prevent confluence-induced differentiation of the cultures, the cells were passaged at 60-70% confluence level.

The culture medium for HCECs was a 1:1 mixture of Ham's F12 and Dulbecco's Modified Eagle's Medium supplemented with 6 (V/V)% heat-inactivated FBS, 1 mM $CaCl_2$, 5 ng/ml human recombinant epidermal growth factor and MycoZap™ Plus-CL. Putative *Mycoplasma* contamination of our cultures was tested regularly with the MycoAlert™ PLUS *Mycoplasma* Detection Kit, and negative result was obtained in every case. Cultivation was carried out in a humidified atmosphere (5% CO_2) at 37°C. In order to exclude possible non-specific effects of the solvents, 1000 times concentrated stock solutions were prepared when using all compounds. These solutions were stored at -20°C or 4°C according to the manufacturers' recommendations. From the stock solutions, the necessary working solutions were prepared immediately before the treatment, using a thousand-fold dilution in the culture medium. The treatments that were to be compared in each experiment were always carried out on the same "vehicle background", and appropriate vehicle control was used. *Cell culture was performed by Ágnes Angyal, Dr. Arnold Markovics, Zsófia Pénzes, Dr. Shahrzad Alimohammadi, Dorottya Horváth, and Dr. József Magi.*

Determination of intracellular lipid content of sebocytes

For the semiquantitative assessment of the sebaceous lipid production, fluorescent Nile Red labeling was used. Cells were plated on 96-well plates for special fluorescence measurements (20 000 cells/well; 24- and 48-hour treatments), and then subjected to appropriate treatments. After removing the supernatant, 100 µl of Nile Red solution in PBS (final concentration: 1 µg/ml) was added to the cells that were incubated for 30 minutes at 37°C. The fluorescence intensity of each well was detected using a FlexStation™ II^{384} or FlexStation 3 multimode plate reader using appropriate wavelengths (excitation wavelength: 485 nm; emission wavelength: 565 nm). *The measurements were performed by Ágnes Angyal, Dr. Arnold Markovics, Dorottya Ádám, Kinga Fanni Tóth, and Dr. József Magi.*

Determination of cellular viability (MTT assay)

Cells were plated in 96-well plates at 20 000 cells/well (sebocytes) or 10 000 cells/well density (corneal epithelial cells), and were subsequently treated with various concentrations of the appropriate substances. After removing the supernatant, 100 µl of MTT solution (final concentration: 0.5 mg/ml) in PBS was pipetted into each well, and incubated at 37°C for 2-3 hours. The MTT solution was then removed, 100 µl "MTT solubilizing solution" was added to each well, and cell-derived formazan crystals were quantitated at 565 nm using the FlexStation 3 plate reader previously mentioned. *The measurements were performed by Ágnes Angyal, Dr. Arnold Markovics, Dorottya Ádám, Kinga Fanni Tóth, and Dr. József Magi.*

Determination of apoptosis and necrosis

Fluorescence measurements of mitochondrial membrane potential were performed with the MitoProbe™ DilC$_1$(5) Assay Kit to investigate apoptosis. In addition to apoptosis, the presence of necrotic processes in the same specimens was examined by simultaneous fluorescent SYTOX Green labeling, which can bind to nuclear DNA in the case of membrane disintegration. The sebocytes were plated at

20 000 cells/well in 96-well plates and treated as indicated. After the supernatant was removed, cells were incubated for 30 min at 37°C with DilC$_1$(5) (1:200) and SYTOX Green (1 µM) diluted in PBS (50 µl/well). At the end of the incubation, cells were washed twice with 100 µl/well PBS and the fluorescence intensity was measured using a FlexStation 3 fluorescent plate reader (DilC$_1$(5) excitation/emission wavelengths: 630/670 nm; SYTOX Green excitation/emission wavelengths: 490/520 nm). Positive controls for apoptosis and necrosis were CCCP (1:200; 37°C for 30 min) and lysis buffer (1:100; 37°C for 30 min), respectively. *The measurements were performed by Ágnes Angyal, Dr. Arnold Markovics, Dorottya Ádám, Kinga Fanni Tóth, and Dr. József Magi.*

Determination of proliferation (CyQUANT proliferation assay)

Cells were plated on 96 well plates at 2 000 cells/well and treated as indicated. The supernatant was then removed and the plates were placed at -80°C until measurement that permeabilized the cells. Subsequently, 200 µl of CyQUANT GR stock solution diluted according to the manufacturer’s protocol was added to each well, and was incubated for 5 minutes at room temperature, protected from light. Fluorescence intensity was detected using a FlexStation 3 (excitation/emission wavelengths: 480/520 nm). *The measurements were performed by Ágnes Angyal, Dr. Arnold Markovics, Dorottya Ádám, Kinga Fanni Tóth, and Dr. József Magi.*

Flow cytometry

SZ95 sebocytes were plated on 6-well plates at 200,000 cells/well density, and treated as indicated. The cells were then scraped in PBS, the suspension was homogenized by intensive trituration, and flow cytometry was performed using a BD FACS Calibur instrument. Cell differentiation status was assessed by detecting side scatter, a parameter that reflects the degree of granularity, and the data were analyzed using *FlowJo V10.4* software. *The measurements were carried out by Ágnes Angyal, Dr. Arnold Markovics, and Zsófia Pénzes.*

UVB irradiation

HCECs were plated at a density of 500,000 cells/Petri dish (d=35 mm). After the attachment of the cells, the culture medium was replaced with 800 μl of colorless Sebomed™ Basal Medium, and the cells were exposed to UVB (λ: 312 nm) irradiation at a dose of 40 mJ/cm^2. The medium was replaced with the conventional culture medium (containing the appropriate active agents or their vehicles) immediately after irradiation. *The measurements were performed by Ágnes Angyal.*

Reverse transcription followed by quantitative real-time polymerase chain reaction (Q-PCR)

Following reverse transcription, quantitative real-time polymerase chain reaction was performed on a Roche LightCycler 480 System using a 5’ nuclease assay. Total RNA was isolated using TRIzol and was subjected to DNase treatment. Subsequently, cDNA was prepared from 1 μg of RNA. The Q-PCR reaction was performed with TaqMan assays. Expression of *18S RNA*, *GAPDH* or *PPIA* was determined as internal control. *The measurements were performed by Ágnes Angyal, Dr. Arnold Markovics, Dr. Erika Lisztes, and Dr. József Magi.*

Western blot

Cells were plated on 96 mm diameter Petri dishes and samples were collected when the appropriate confluence level was reached or after the indicated treatments. Upon sample collection, cells were plated in a “detergent mix” containing a protease inhibitor cocktail and PhosSTOP reagent. Ultrasonic disruption was then performed on ice using a sonicator. Protein concentration was determined by BCA protein assay and adjusted to a uniform 1 mg/ml. Equal amounts (10 μg) of the protein samples were subjected to SDS polyacrylamide gel electrophoresis. The separated proteins were transferred to nitrocellulose membranes. The membranes were then incubated overnight at 4°C with primary antibodies diluted in the blocking solution for 1 h in 50 mg/ml milk powder containing TBST at room temperature to block the free binding sites. The secondary antibodies were also diluted in blocking solution (incubation: 37°C, 1 hour). The immunosignals were visualized with SuperSignal® West Pico Chemiluminescent Substrate kit by using a KODAK Gel Logic 1500 Imaging System. Semi-quantitative densitometric analysis of chemiluminescent signals was performed using Fiji software. *The measurements were performed by Ágnes Angyal, Dr. Arnold Markovics, Kinga Fanni Tóth and Dr. József Magi.*

Phosphokinase array

In order to identify those kinase cascades that were activated by OEA treatment, the Proteome Profiler Human Phospho-Kinase Array Kit was used according to the manufacturer’s protocol. Sebocytes were seeded in Petri dishes at a density of 10 million cells/15 ml culture medium. Samples were collected as described for Western blotting, and their protein content was determined. Equal amounts of protein samples were added to the membranes, and chemiluminescent signals were detected with the KODAK Gel Logic 1500 Imaging System using the same settings for each membrane. Semi-quantitative densitometric analysis of the signals was performed using Fiji software. *The experiments were performed by Ágnes Angyal, Dr. Arnold Markovics, and Kinga Fanni Tóth.*

Immunohistochemical detection of GPR119

The donors gave their written consent to the use of the samples for research purposes after having been duly informed. The experiments were approved by the Regional and Institutional Research Ethics Committee of the University of Debrecen and the Hajdú-Bihar County Government Office (IDs: IX-R-052/01396-2/2012, IF-

12817/2015, IF-1647/2016, IF-778-5/2017, DE RKEB/IKEB 4988-2018) in compliance with the guidelines of the Declaration of Helsinki.

In situ immunohistochemical detection of GPR119 was performed on formalin-fixed, paraffin-embedded, sebaceous gland-rich areas of skin samples from 3 donors diagnosed with trichilemmal cysts. Sections were prepared from the paraffin-embedded blocks and heat-induced antigen retrieval was performed on the sections in citrate buffer in a pressure cooker under maximum pressure. 3% H_2O_2 solution was used for 10 min to inhibit endogenous peroxidases. Sections were then incubated at room temperature with a primary antibody recognizing human GPR119 diluted in TBS containing BSA. Secondary immunolabeling and visualization were performed using “EnVision FLEX Labeled polymer-HRP anti rabbit and anti-mouse System” and DAB. Nuclei were labeled with hematoxylin and sections were covered with appropriate coverslips. *The experiments were performed by Dr. Ágnes Pór and Dr. Ilona Kovács.*

Semi-quantitative analysis of the expression of GPR119 in acne

GPR119 expression patterns were examined in skin samples from 6 donors with and 6 donors without acne who received no anti-acne medications prior to the study. Sections were prepared from paraffin-embedded blocks and heat-induced antigen retriveal was performed on the sections. 3% H_2O_2 solution was used for 10 min to inhibit endogenous peroxidases. Sections were then incubated at room temperature with a primary antibody recognizing human GPR119 diluted in BSA containing PBS. For the secondary immunolabeling, as well as for the visualization we used the protocol described in the previous chapter. It is important to emphasize that the processing and staining of the sections involved in the semi-quantitative comparison were performed simultaneously, in a strictly standardized manner, and the samples were photographed by using identical settings (brightness, white balance, etc.). *The* *samples were provided by Dr. Dániel Törőcsik, the experiments were performed by Dr. Ágnes Pór and Dr. Ilona Kovács, and the image analysis was performed by Dr. Attila Oláh.*

Detection of ECS members on HCECs (immunofluorescent labeling)

HCECs were grown on coverslips to 60-70% confluence and fixed in acetone at -20°C for 10 min. Non-specific binding sites were blocked and cells were permeabilized in 0.6% Triton X-100 and 10 mg/ml BSA containing PBS (5 min, room temperature). Cells were incubated overnight at 4°C with primary antibodies against FAAH, CB_1, NAPE-PLD, MAGL, CB_2, DAGLα and DAGLβ. After washing in PBS, the coverslips were incubated with Alexa-488®-conjugated secondary antibodies against mouse IgG Fc segment or rabbit IgG Fc segment for 1 hour at room temperature. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Labeling with omission of primary antibodies was used as a negative control. Images were taken with an Olympus Xcellence RT fluorescence microscope. *The experiments were performed by Ágnes Angyal.*

Detection of ECS members on human cornea (immunofluorescent labeling)

The use of corneal samples from deceased persons was approved by the Regional and Institutional Research Ethics Committee of the University of Debrecen and the Hajdú-Bihar County Government Office (IDs: IX-R-052/00016-28/2012; DE OEC RKEB/IKEB 3580-2012) in accordance with the guidelines of the Declaration of Helsinki. Sections were fixed in acetone, blocked and permeabilized in 0.1% Triton X-100 and 10 mg/ml BSA containing PBS, and incubated overnight at 4°C with antibodies diluted in the blocking solution described in the previous subsection. The slides were then washed with PBS and incubated with Alexa-568®-conjugated secondary antibodies against mouse IgG Fc-segments produced in goats and rabbit IgG Fc-segments also produced in goats. Nuclei were counterstained with

DAPI, and sections were covered with Fluoromount-G. Images were taken with a Zeiss LSM 880 fluorescence microscope. In all cases, negative controls were obtained by omitting the primary antibodies. *Human cornea samples were provided by Dr. Lili Takács, and experiments were performed by Ágnes Angyal, Dr. Barbara Zsebik and Prof. Dr. György Vereb.*

Determination of intracellular 3'5'-cyclic adenosine monophosphate (cAMP) concentration (cAMP ELISA)

SZ95 sebocytes were treated for 1 h with OEA (50 μM) or an equal volume of solvent (absolute ethanol). Cells were then lysed at a density of 10^7 cells/ml following the manufacturer’s protocol and the intracellular cAMP level was assayed using Parameter Cyclic AMP Assay kit following the manufacturer's protocol. *The experiments were performed by Ágnes Angyal.*

Cytokine release assay (IL-6 and IL-8 ELISA)

In the cytokine release assay, cells plated in a standardized manner (500 000 cells in 1.5 ml medium, 35 mm diameter Petri dishes) were treated as indicated for 3 or 24 hours. The supernatants were then collected, centrifuged (500 g; 10 min) and the debris-free supernatant was stored at -80°C. The amount of IL-6 and IL-8 was determined using OptEIA kits according to the manufacturer’s protocol. *The measurements were performed by Ágnes Angyal, Dr. Arnold Markovics, Kinga Fanni Tóth, Zsófia Pénzes, Dr. Shahrzad Alimohammadi and Dorottya Horváth.*

Fluorescence Ca^{2+} measurement

To study the effects of OEA on the Ca^{2+} homeostasis, fluorescent Fluo-4 AM labeling was used. Cells were cultured at 20,000 cells/well density in special 96-well plates optimized for fluorescence measurements. Cells were washed once with 100 μl/well Hank's solution supplemented with probenecid and BSA (final concentrations: 2.5 mM and 10 mg/ml, respectively), and incubated with Hank's solution containing 1 μM Fluo-4 AM dye (100 μl/well) for 30 min at 37°C. The cells were then washed three times with Hank's solution (100 μl/well) and the fluorescence intensity of each well was determined in “Flex” mode using a FlexStation 3 device ($\lambda_{ex/em}$: 490/520 nm). As a positive control, at the end of each measurement ATP was pipetted to each well (final concentration: 0.2 mg/ml). The data were expressed as F/F_0, where F_0 is the mean baseline fluorescence intensity measured before treatment and F is the actual fluorescence. *Measurements were performed by Ágnes Angyal, Dr. Arnold Markovics, and Kinga Fanni Tóth.*

Selective gene silencing (siRNA transfection)

SZ95 sebocytes were plated in Petri dishes, 96-well plates, or sterile coverslips in 6-well dishes, and the next day (at 50-70% confluence) the medium was replaced with serum-free OptiMem medium, and cells were transfected with GPR119-specific small interfering RNA (siRNA) oligonucleotides using Lipofectamine® RNA_i MAX transfection reagent. As a control, cells were transfected with Stealth RNA_i Negative Control “medium” double-stranded siRNA, which does not show homology to any known mRNA sequence. The efficiency of gene silencing was checked at mRNA (Q-PCR) and protein level (Western blot) on days 2 and 3 after transfection. *Measurements were performed by Ágnes Angyal and Dr. Arnold Markovics.*

Statistical analysis

Data were analyzed using *IBM SPSS Statistics 19* or *GraphPad Prism 8.3.1* software. Data were evaluated with Student’s two-tailed, unpaired t-test (pairwise comparisons), or Bonferroni *post hoc* tests (multiple comparison) following one-way ANOVA. $p<0.05$ values were considered as a significant differences in all cases. The graphs were plotted using *Origin Pro Plus 6.0* or *GraphPad Prism 8.3.1* software. *The analysis and the preparation of the figures were carried out by Ágnes Angyal, Dr. Arnold Markovics, Dr. Attila Gábor Szöllősi and Dr. Attila Oláh.*

Results

OEA promotes differentiation of the sebocytes

The "classical" eCBs (e.g., AEA), as well as the inflammatory lipid mediator arachidonic acid (AA), typically increase sebaceous lipid production at concentrations of 30-50 μM over 24-48-hr of treatments. Based on this, the effects of OEA were also first investigated in the concentration range between 10 nM and 50 μM. We found that, up to 50 μM, OEA did not affect viability (24-48-hr treatments; MTT assay), nor did it affect SZ95 sebocyte proliferation (CyQUANT assay; 48-hr treatments). Conversely, OEA significantly increased sebaceous lipid production in a concentration-dependent manner (1-50 μM; 24-48-hr treatments; Nile Red labeling) and also increased granularity of sebocytes (48-hr treatments; flow cytometry). These results suggested that, similar to "classical" eCBs, OEA is able to promote sebocyte differentiation, and its lipogenic effects are comparable to those induced by "classical" eCBs (e.g., AEA), AA, and the combination of linoleic acid and testosterone. Considering that in addition to increased lipid production and granulation, as a characteristic of holocrine secretion, the differentiation of sebocytes is also accompanied by cell death processes, we asked whether OEA treatment affects the ratio of apoptotic or necrotic cells. Based on our results obtained by combined DilC$_1$(5)-SYTOX Green labeling, 48-hr OEA treatment slightly, but significantly decreased mitochondrial membrane potential (DilC$_1$(5) labeling), indicating the initiation of early apoptotic processes, while the proportion of necrotic cells remained unchanged (SYTOX Green labeling). This result confirmed that OEA treatment indeed induced differentiation of human sebocytes.

OEA induces an inflammatory response in human sebocytes

In order to further investigate the effects of OEA on human sebocytes, we examined its effects on the immunophenotype of the cells. We found that 3-hr OEA treatment (50 μM) significantly increased the expression of several key inflammatory cytokines (IL-1α, IL-1β, IL-6 and IL-8) at the mRNA level (Q-PCR) and increased the release of IL-6 and IL-8 (ELISA), although the effect was generally below that observed with Toll-like receptor 4 activator LPS used as a positive control. Thus, our data suggest that in addition to promoting differentiation, OEA can also induce a significant inflammatory response in human sebocytes.

Lipogenic effect of OEA is not mediated via the PPARα pathway

We then investigated what might underlie the differentiation promoting effects described above. It is known from the literature that PPARs are important players in the regulation of lipid synthesis, and are also expressed in human sebocytes. Since OEA is able to activate PPARα, we investigated whether this nuclear receptor is involved in mediating its lipogenic effect on human sebocytes. We found that the selective PPARα antagonist GW 6471 (1 μM) did not significantly affect OEA-induced sebaceous lipid production (48-hr treatments), suggesting that, in human sebocytes, the differentiation and sebaceous lipid production enhancing effect of OEA is most likely mediated through a PPARα-independent pathway.

GPR119 is expressed on human sebocytes *in vitro* and *in situ*

Because according to the literature data not only PPARα, but also GPR119 (a recently de-orphanized G-protein coupled receptor) may also mediate the biological effects of OEA, we investigated whether this receptor is expressed in human sebocytes. We have shown that GPR119 was present at both the mRNA (Q-PCR) and protein levels (Western blot) in cultured human sebocytes and was also expressed *in situ* in human sebaceous glands (immunohistochemistry).

Selective gene silencing of GPR119 reduces the lipogenic effect of OEA

Having demonstrated by several methods that GPR119, a putative receptor for OEA, was expressed on human sebocytes, we investigated whether it played a role in mediating the biological effects of OEA. In the lack of known selective antagonists, this was investigated using siRNA-mediated selective gene silencing. We found that although the reduction of GPR119 expression (monitored by Q-PCR at the mRNA level and by Western blot at the protein level) could not completely abrogate it, it was able to significantly suppress the lipogenic effect of OEA as compared to that observed in SCR control cells transfected with non-sense RNA. This indicates that activation of GPR119 is likely to play a role in the development of the lipid synthesis enhancing effect of OEA, but it cannot be excluded that GPR119-independent lipogenic pathways may also contribute to this process.

Receptor-mediated effects generally exhibit saturation kinetics. Thus, in order to indirectly confirm these results, we also examined the effects of higher concentrations of OEA (100, 300 and 500 μM) on the lipid synthesis. We found that sebaceous lipid synthesis was further enhanced by higher (100 and 300 μM) OEA concentrations, where the effect was saturated, as lipid production following treatment with 100 and 300 μM did not differ from each other significantly. Obviously, application of the clearly cytotoxic concentration (500 μM) of OEA resulted in a lower lipid level.

Several kinase cascades are involved in the lipogenic effect of OEA

Next, we investigated the effects of OEA on some regulators that are known to be important for sebaceous lipid production, such as Ca^{2+} homeostasis and the ERK1/2 MAPK cascade. We found that acute OEA treatment (50 μM) did not affect $[Ca^{2+}]_{IC}$ (Fluo-4 AM fluorescent Ca^{2+} measurement), but significantly enhanced ERK1/2 MAPK phosphorylation (Western blot), i.e., similar to the “classical” eCBs, it also activated the cascade. Considering that inhibition of ERK1/2 MAPK significantly reduced sebaceous lipid production in case of “classical” eCBs, we next investigated how the ERK1/2 MAPK inhibitor PD 98059 affects the lipogenic effect of OEA. We found that co-administration of PD 98059 significantly reduced the lipogenic effect of OEA, confirming that activation of this pathway is indeed involved in mediating the effects of OEA. However, it should also be noted that, when applied alone, PD 98059 also reduced basal sebaceous lipid production, indicating that the “basal”, resting activity of the pathway probably also contributes to the maintenance of homeostatic sebaceous lipid production of the sebocytes. Since GPR119 exerts its biological effects generally (although not exclusively) as a G_s protein-coupled receptor, we also investigated whether OEA affects intracellular cAMP levels in human sebocytes. Interestingly, we found that OEA treatment (50 μM; 60 min) only slightly and non-significantly increased cAMP levels in human sebocytes.

In order to obtain further information on the exact mechanism of the cellular effects of OEA, we used the “Proteome Profiler Human Phospho-Kinase Array Kit” to investigate which kinase cascades are activated in human sebocytes by OEA treatment. We found that, among others, the CREB, STAT5a/b, JNK 1/2/3, and Akt/PKB signaling pathways were activated by OEA treatment (20 min; 50 μM). Subsequently, the involvement of the most relevant pathways (i.e., JNK, Akt/PKB, CREB, and STAT5) in mediating the effects of OEA was also investigated using specific inhibitors. We found that pharmacological inhibition of STAT5 did not significantly alter OEA-induced lipogenesis, whereas selective inhibition of JNK, CREB and Akt/PKB significantly reduced it.

GPR119 is down-regulated in acne

The above data suggest that dysregulation of homeostatic OEA→GPR119 signaling may contribute to the development of pathologies characterized by abnormal sebaceous gland function, such as seborrhea or acne. Therefore, to increase the translational relevance of our experiments, we decided to investigate the putative alterations in GPR119 expression in sebaceous glands obtained from donors suffering from acne as well as from acne-free individuals. Following a standardized immunohistochemical assay and semi-quantitative image analysis, we found that despite the low numbers of the donor and the relatively high inter-donor variability, GPR119 expression was significantly reduced in sebaceous glands of acne patients. This implies that disruption of homeostatic GPR119 signaling may occur in at least some of the acne patients.

Human corneal epithelial cells express key members of the ECS

In the second half of our experiments, we investigated the presence of the most important members of the ECS, i.e. the "classical" cannabinoid receptors (CB_1 and CB_2) and the enzymes that synthesize (NAPE-PLD, DAGLα and -β) and degrade (FAAH and MAGL) eCBs, in human corneal epithelial cells *in vitro* and *in situ*. Immunofluorescent labeling confirmed that HCECs express CB_1 and CB_2, as well as key enzymes (see above) involved in the metabolism of the eCBs. It is important to note that the same proteins were also found in human corneal epithelium *in situ*. This therefore implies that human corneal epithelium may indeed be capable of eCB production and degradation. The eCB tone regulated by both processes may play a role in, among other things, fine-tuning local inflammatory processes.

The inflammatory response induced by TLR3 activation and UVB irradiation exhibits a characteristic time-dependence in HCECs

Subsequently, we aimed to investigate whether the previously reported CB_1-dependent anti-inflammatory effect of AEA is restricted to the suppression of the TRPV1 activation-induced inflammatory responses, or it is a universal effect that remains effective in case of TRPV1-independent inflammatory signals. We first used two clinically relevant inflammation inducers acting through mechanisms independent of TRPV1 and independent of each other, namely p(I:C) (20 μg/ml;

mimicking viral keratitis through TLR3 activation), and UVB irradiation (40 mJ/cm^2) as a model of photodamage-induced keratitis.

First, we examined the dynamics of the inflammatory response induced by the two treatments. We found that both stimuli increased the expression of several inflammatory cytokines (IL-1α, IL-1β, IL-6 and IL-8) at the mRNA level. While analyzing the time-course of the action (time points examined in case of p(I:C) are 3, 6, 12, and 24 hrs; time points examined in case of UVB are 3, 6, 12, 24, and 48 hrs), we found that the elevation was most pronounced at 3 hrs (p(I:C) treatment) and at 12 hrs (UVB irradiation). Thus, we also assessed the release of inflammatory cytokines into the supernatant (ELISA) at these time points. We found that IL-6 and IL-8 levels were increased in both cases, while IL-1α and IL-1β concentrations were below the detection limit (unpublished observation). In the light of these results, further experiments were performed using 3- (TLR3 activation) and 12-hr treatments and incubation times (UVB model).

AEA effects on the expression and release of inflammatory cytokines show significant concentration and model dependence in HCECs

To investigate the potential anti-inflammatory effects of AEA on human corneal epithelial cells, AEA was applied at low (100 nM) and high (10 μM) concentrations in the above inflammatory models. First, we showed that AEA did not significantly affect the viability of HCECs at the indicated concentrations, i.e., it could be used without risk of cytotoxicity. Surprisingly, when applied alone, both concentrations of AEA caused a significant increase in the mRNA level expression of the inflammatory cytokines (3-hr treatments), and this increase was still present after 12 hours in case of IL-6 and IL-8 (both concentrations) and IL-1α (10 μM). Interestingly, in spite of its pro-inflammatory effect, 100 nM AEA treatment significantly decreased the effect of p(I:C) on IL-6, IL-8, and IL-1β expression. At

10 μM, however, the opposite effect was observed, as AEA caused a further increase in IL-6 and IL-1β levels, while having no effect on IL-8 and IL-1α expression. In contrast to the TLR3 activation model, no anti-inflammatory effect was observed after UVB irradiation (40 mJ/cm^2) (12-hr treatment). In fact, at 10 μM, AEA further increased IL-6 and IL-8 expression, but did not affect the mRNA level of IL-1α and -β, and even 100 nM of AEA increased IL-8 levels. We also wanted to investigate the most striking changes at the protein level, hence we assessed IL-6 and IL-8 in the supernatants using ELISA technique. As expected, both p(I:C) and UVB irradiation significantly increased the concentrations of IL-6 and IL-8 in the supernatant. Interestingly, this was further increased by both concentrations of AEA. In contrast, when we examined the effects of AEA alone, we found that at 100 nM, it significantly reduced spontaneous IL-8 release after both 3- and 12-hr treatments, but had no effect on IL-6 concentrations. In contrast, at 10 μM, it increased IL-8 at both time points, and significantly increased IL-6 release in case of 3-hr treatments.

Discussion

In the first half of our experiments, we investigated previously unexplored aspects of the ECS in human sebaceous glands. Previously, we have shown that sebocytes are capable of metabolizing OEA. This shed light to the possibility that, in addition to the “classical” eCBs described previously (i.e., AEA and 2-AG), this “eCB-like” molecule may also influence the biological processes of human sebaceous glands.

Investigation of “non-classical” eCB signaling in human sebaceous glands

To clarify the possible role of OEA, we first used complementary cell physiology assays, and found that this molecule promoted differentiation of human sebocytes, significantly enhanced sebaceous lipid production, increased the granularity of the sebocyte, and induced early apoptotic processes. To further investigate the biological effects of OEA, we also showed that potent lipogenic concentrations (50 μM) induced an inflammatory response in sebocytes, but did not affect viability or cell count (24- and 48-hr treatments).

Having the basic phenomenon described, we continued our experiments by studying the mechanism of action. We have shown that the lipogenic effect of OEA was independent of PPARα, and therefore investigated whether another possible receptor for OEA, namely GPR119, was expressed on sebocytes. We found that GPR119 was present on cells at both mRNA and protein levels and was also expressed in intact sebaceous glands. In the absence of selective antagonists, we next successfully reduced the expression of GPR119 in human sebocytes by siRNA transfection, and demonstrated that the intervention significantly attenuated the lipogenic effect of OEA, which was most likely (at least in part) mediated by GPR119 activation.

In order to further investigate the mechanism of the lipogenic effect of OEA, we used a phosphokinase array ("screening") as well as cAMP ELISA. We found that not only the ERK1/2 MAPK cascade (a known lipogenic pathway for "classical" eCBs), but also the activation of other signaling pathways (JNK, Akt/PKB and CREB) were involved in mediating lipogenic effects of OEA, whereas pharmacological inhibition of STAT5 (a signaling molecule involved in regulating immune processes of the pilosebaceous unit) had no significant effect on it. It is important to note that OEA had no effect on the Ca^{2+} homeostasis of the sebocytes (50 μM; acute treatment). Moreover, although activation of GPR119 typically increases intracellular cAMP levels via a G_s-protein coupled pathway, interestingly, we found that it only increased the cAMP concentration of the sebocytes in a small, non-significant extent (50 μM; 60 min treatment). This latter result may be explained by the activation of alternative second messenger pathways (biased agonism). On the other hand, however, considering the well-known compartmentalization of cAMP signaling, it does not exclude the possibility that cAMP may contribute to some of the effects of OEA. Indeed, in our experiments, we determined cAMP levels from whole cell lysates (and only at a single time point), but did not test whether cAMP levels in different intracellular microcompartments (and/or at other time points) are statistically significantly increased by OEA treatment. Taken together, our results from the phosphokinase array (namely that CREB is phosphorylated [activated] by OEA treatment and that pharmacological inhibition of CREB significantly reduces the lipogenic effect of OEA) suggest that cAMP may (also) play a role in mediating some of the effects of OEA on human sebocytes. It is important to note, however, that CREB activation may not only be achieved through an increase in cAMP levels, but also through alternative second messenger pathways, including the Akt/PKB pathway.

It is well-known that Akt/PKB as well as JNK MAPK pathways can mediate lipogenic effects of various mediators on human sebocytes. Consistent with this, our results showed that OEA treatment activated these pathways, and their pharmacological inhibition reduced OEA-induced sebaceous lipid production.

To conclude our experiments on the OEA-GPR119 axis, we compared GPR119 protein expression in skin samples of acne patients and acne-free donors. We found that GPR119 protein expression was significantly reduced in sebaceous glands of acne patients. Although, due to the small number of the donors and the relatively high inter-individual variability, it would be premature to draw bold conclusions, our data suggest that homeostatic GPR119 signaling may indeed be impaired in a subset of acne patients. Although our experiments do not answer the question why the pro-lipogenic GPR119 is down-regulated in the sebaceous glands of patients with acne, theoretical considerations suggest several possible explanations. Down-regulation/desensitization following activation is a well-known phenomenon for several G protein-coupled receptors, including GPR119. In light of this, one possible explanation for our results is that (over)activation of GPR119 plays a role in the initial stage of acne pathogenesis rather than in its subsequent progression. An alternative hypothesis may be that down-regulation of the lipogenic GPR119 receptor is a secondary compensatory mechanism initiated in response to the already abnormally high levels of sebum production.

GPR119 is known to be a potential therapeutic target in the future as an adjunctive treatment for type 2 diabetes mellitus. Although the synthetic agonists investigated have not progressed beyond phase II clinical trials so far, promising animal data still encourage the development and testing of new GPR119 activators with greater *in vivo* efficacy. Given that different agonists of GPR119 may activate different second messenger pathways, our results do not necessarily imply that administration of all GPR119 agonists will be associated with dermatological side effects or impact on sebaceous gland function. However, for agonists that activate second messenger pathways that overlap to a large extent with OEA, the development of such side effects cannot be excluded (provided, of course, that they reach sufficiently high concentrations in the sebaceous glands). In the light of the experimental data we have now reported, it will therefore be worth paying attention to the development of potential dermatological complications in future clinical trials.

Overall, our results identify the OEA-GPR119 signaling pathway as a previously unknown positive regulator of human sebaceous lipid production and sebocyte differentiation. OEA treatment of cells led to the development of a pro-inflammatory phenotype, raising the possibility that dysregulation of this signaling system may contribute to the development of acne characterized by increased sebaceous lipid production and inflammation.

Investigation of ECS in HCECs

The epithelial cells that build up the external layer of the cornea act as the first line of defense for the surface of the eye, and hence must also handle physical and immunological challenges. These latter challenges are of great importance, as pathological inflammatory processes in the cornea can lead to loss of corneal transparency and thus blindness. Recent findings suggest that HCECs are not only “passive sufferers”, but also active regulators of local inflammatory processes.

TRPV1 is one of the a regulators of the inflammatory responses of HCECs, as activation of this receptor leads to the release of inflammatory mediators. Given that the major “classical” eCBs (AEA and 2-AG) are present in the human corneal epithelium, in the current study, our aim was to explore the expression of previously unidentified members of the ECS in human corneal epithelium, and to investigate whether AEA also exerts anti-inflammatory effects in inflammatory processes that are independent of TRPV1 activation.

First, we confirmed (CB_1 and CB_2) and newly demonstrated (NAPE-PLD, DAGLα and -β, MAGL, FAAH) that key members of the ECS are expressed in cultured HCECs as well as in intact human corneal epithelium. We then examined the potential anti-inflammatory role of AEA in more detail. To this end, we selected two clinically relevant stressors (TLR3 activation to mimic viral infections and UVB irradiation) whose inflammation-inducing effects are likely to be independent of TRPV1 activity. We have shown that both p(I:C) treatment and UVB irradiation enhance the mRNA expression of various inflammatory cytokines (IL-1α, IL-1β, IL-6, IL-8) in HCECs with a characteristic time course. Having shown that the selected test concentrations (100 nM and 10 μM) of AEA do not affect the viability of HCECs, we first observed the effects of AEA administered alone. Surprisingly, we found that AEA increased the mRNA expression of several inflammatory cytokines at both concentrations and both time points. In addition, although it reduced spontaneous IL-8 release in course of both 3- and 12-hr treatments at 100 nM, it enhanced it at both time points when applied at 10 μM. Moreover AEA significantly increased IL-6 levels in course of 3-hr treatments. Thus, contrary to our expectations, it was found to be predominantly pro-inflammatory. Our data also revealed that AEA can differentially modulate *de novo* production and release of pre-existing stored cytokines. We also showed that, although, when applied at 100 nM, AEA reduced the p(I:C)-induced mRNA expression of several inflammatory cytokines, it further enhanced IL-6 and IL-8 release. Moreover, when applied at 10 μM, it was also clearly pro-inflammatory. When examining the UVB model, we found an even more consistent biological behavior: in all cases when AEA had a statistically significant effect, it was a further enhancement of the inflammatory response.

At first glance, these results seem to contradict literature data. Indeed CB_1 activation by a synthetic agonists or AEA were reported to inhibit the inflammatory response induced by TRPV1 activation. Although the underlying mechanism of this phenomenon has not been investigated in detail, several hypotheses can be formulated to resolve the discrepancy. First of all, it is important to note that AEA itself can also activate TRPV1. Thus, it is possible that AEA is only anti-inflammatory on corneal epithelial cells, if TRPV1 is activated by a “stronger” ligand with higher efficacy, in which case AEA may interfere with the effect as a “weaker”, partial agonist. Because in our experiments no such a ligands were administered, AEA might have become pro-inflammatory because of direct activation of TRPV1, and this inflammatory response was additive with inflammatory processes induced by an independent mechanism. Of course, when investigating the background of this phenomenon, it is also worth considering that AEA may also act on a number of other molecular targets (e.g., GPR18, GPR55, several calcium channels, etc.). Whether or not any of these (already known or yet to be identified) alternative targets are expressed on corneal epithelial cells and influence their inflammatory processes is to be explored in future targeted experiments.

Finally, it should also be noted that according to our results, FAAH (i.e., the main catabolic enzyme responsible for the degradation of AEA) was also expressed on human corneal epithelial cells *in vitro* and *in situ*, and that the degradation of AEA by FAAH results in the production of a pro-inflammatory lipid mediator, arachidonic acid. Arachidonic acid and its derivatives (e.g., leukotriene B4) are important players in the pathogenesis of a wide range of ophthalmic diseases, including many corneal pathologies, to the extent that their inhibition has recently emerged as a promising new therapeutic tool in the mitigation of some ocular inflammatory processes. In light of this, it is possible that, under certain conditions, AEA is rapidly metabolized to arachidonic acid and then to other pro-inflammatory mediators, triggering the inflammatory response observed in our experiments, which, for some yet unknown reason, did not occur in the presence of TRPV1 activators. Of course, our current knowledge is not sufficient to determine which of the above mechanisms may contribute to the inflammatory effects of AEA described here, and further studies are needed to better understand the process.

In any case, a better understanding of the role of ECS in the pathomechanism of various keratitides and other inflammatory eye diseases may lead to the development of effective new therapeutic tools for these diseases. However, the results of our present study also point to the need for caution when intervening in such a complex system, as even eCBs, albeit commonly known as anti-inflammatory agents, can exert pro-inflammatory effect.

Summary

In the first part of our experiments, we investigated the effect of OEA on human sebocytes. Up to 50 μM, OEA could be used without the risk of cytotoxicity, and it did not affect the proliferation of SZ95 sebocytes either. Using a variety of assays, we also showed that OEA could increase sebaceous lipogenesis as well as differentiation of sebocytes, and it increased the expression and release of several pro-inflammatory cytokines, too. While further exploring these phenomena, we found that OEA did not exert its lipogenic effect by activating PPARα, as it was not affected by selective PPARα antagonism. Next, we demonstrated that human sebocytes expressed GPR119, another potential receptor for OEA, and selective gene silencing of the said receptor significantly suppressed the lipogenic effect of OEA. Moreover, we could also show that the development of the lipogenic effect depended on the activation of several kinase cascades (ERK1/2, JNK, CREB, Akt/PKB). Finally, assessment of the expression of GPR119 in the sebaceous glands of acne patients as well as of acne-free individuals demonstrated that GPR119 was down-regulated in acne, suggesting that the OEA-GPR119 signaling axis may indeed be disturbed during the pathogenesis of the disease. In the second part of our experiments, we investigated HCECs as well as human corneal samples, and found that human corneal epithelial cells expressed major members of ECS *in vitro* and *in situ*. We also showed that, contrary to expectations, AEA treatment of HCECs led to a proinflammatory effect. Moreover, albeit its effects were concentration-dependent, in most of the cases AEA further enhanced the inflammatory response evoked by TLR3 activation (mimicking viral keratitis) or by UVB irradiation (mimicking photodamage). Thus, our results suggest that eCB signaling, albeit generally considered to exert anti-inflammatory effects, may mediate proinflammatory actions in the human corneal epithelium under certain conditions.

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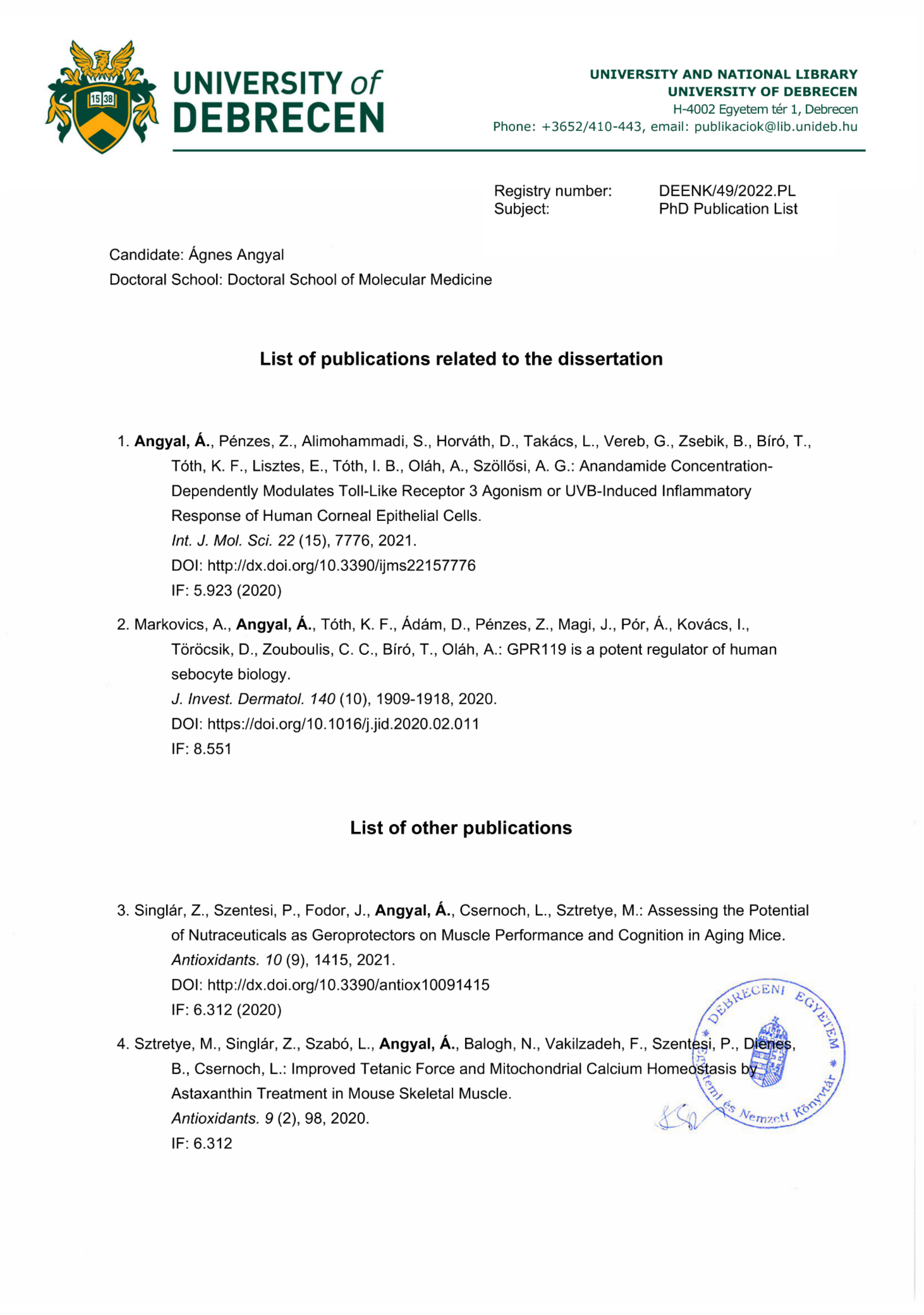
Appendix – List of publications



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Subject: PhD Publication List

Candidate: Ágnes Angyal
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Angyal, Á.**, Pénzes, Z., Alimohammadi, S., Horváth, D., Takács, L., Vereb, G., Zsebik, B., Bíró, T., Tóth, K. F., Lisztes, E., Tóth, I. B., Oláh, A., Szöllősi, A. G.: Anandamide Concentration-Dependently Modulates Toll-Like Receptor 3 Agonism or UVB-Induced Inflammatory Response of Human Corneal Epithelial Cells.
   Int. J. Mol. Sci. 22 (15), 7776, 2021.
   DOI: http://dx.doi.org/10.3390/ijms22157776
   IF: 5.923 (2020)
2. Markovics, A., **Angyal, Á.**, Tóth, K. F., Ádám, D., Pénzes, Z., Magi, J., Pór, Á., Kovács, I., Töröcsik, D., Zouboulis, C. C., Bíró, T., Oláh, A.: GPR119 is a potent regulator of human sebocyte biology.
   J. Invest. Dermatol. 140 (10), 1909-1918, 2020.
   DOI: https://doi.org/10.1016/j.jid.2020.02.011
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List of other publications

3. Singlár, Z., Szentesi, P., Fodor, J., **Angyal, Á.**, Csernoch, L., Sztretye, M.: Assessing the Potential of Nutraceuticals as Geroprotectors on Muscle Performance and Cognition in Aging Mice.
   Antioxidants. 10 (9), 1415, 2021.
   DOI: http://dx.doi.org/10.3390/antiox10091415
   IF: 6.312 (2020)
4. Sztretye, M., Singlár, Z., Szabó, L., **Angyal, Á.**, Balogh, N., Vakilzadeh, F., Szentesi, P., Dienes, B., Csernoch, L.: Improved Tetanic Force and Mitochondrial Calcium Homeostasis by Astaxanthin Treatment in Mouse Skeletal Muscle.
   Antioxidants. 9 (2), 98, 2020.
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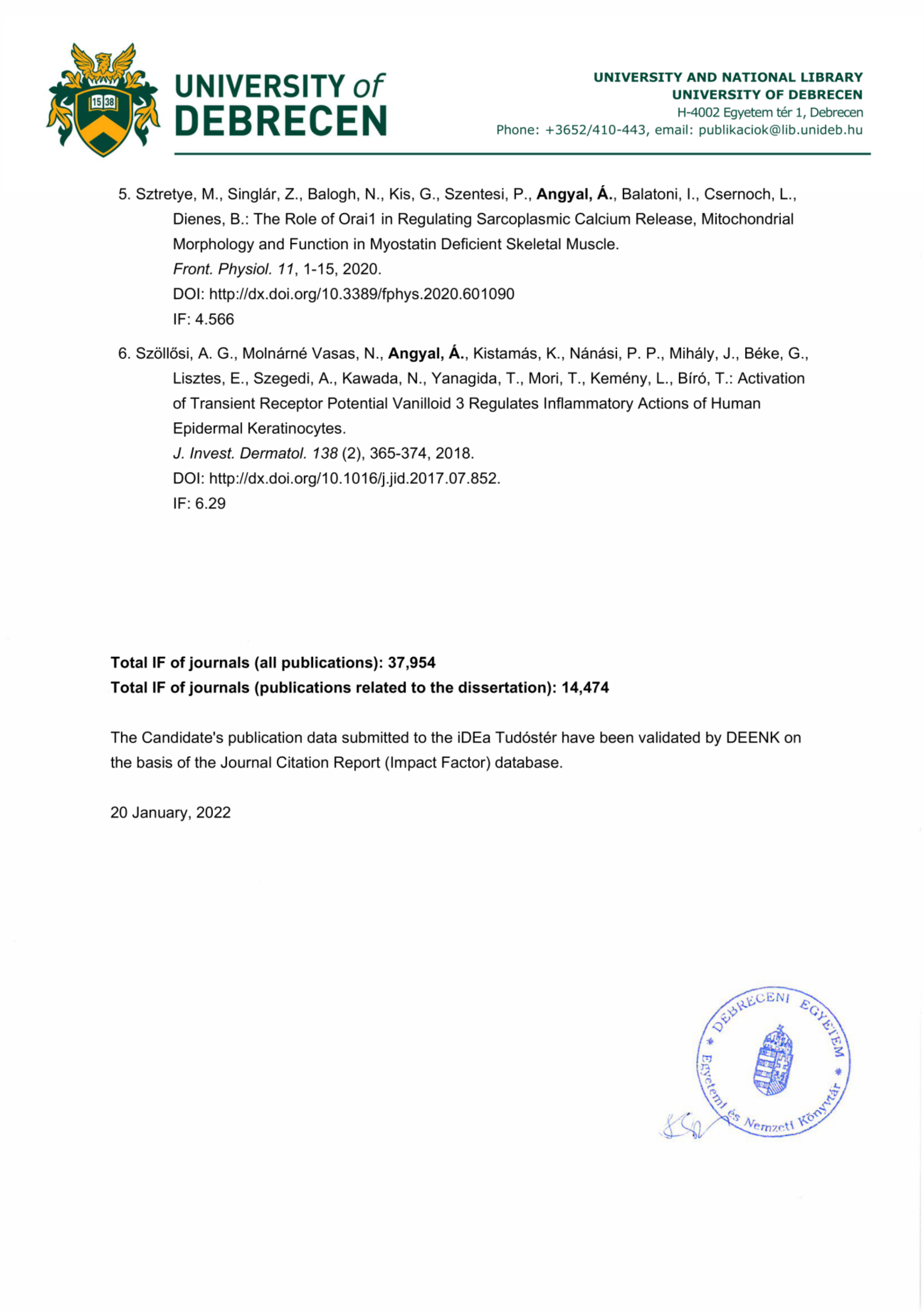

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5. Sztretye, M., Singlár, Z., Balogh, N., Kis, G., Szentesi, P., **Angyal, Á.**, Balatoni, I., Csernoch, L., Dienes, B.: The Role of Orai1 in Regulating Sarcoplasmic Calcium Release, Mitochondrial Morphology and Function in Myostatin Deficient Skeletal Muscle.
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   J. Invest. Dermatol. 138 (2), 365-374, 2018.
   DOI: http://dx.doi.org/10.1016/j.jid.2017.07.852.
   IF: 6.29

Total IF of journals (all publications): 37,954
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