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

Role of cannabinoid signaling in selected (patho)physiological processes of the human skin

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Members of the Examination Committee:	Zoltán Benyó, MD, PhD, DSc
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The Examination takes place at the Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen
February 29, 2016, 11:00

Head of the **Defense Committee**:	János Szöllősi, PhD, DSc
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The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen
February 29, 2016, 14:00

INTRODUCTION AND OVERVIEW OF THE LITERATURE

The human skin and the complex epidermal barrier (CEB)

The skin is one of our largest organs. Its most important role is to demarcate and to protect our body against various environmental challenges by forming the complex epidermal barrier (CEB), but it also has several additional sensory, endocrine (*in situ* hormone production) and other functions (storage, transport, thermoregulatory and exocrine functions).

The first line defense of the CEB is the physicochemical barrier, which is formed as the result of the differentiation of the epidermal keratinocytes. At the end of their development these cells undergo apoptosis, lose their nuclei, and, being embedded in a special extracellular matrix as "bricks in the mortar", compose the primary "external interface" of our body. Finally, impregnation of this surface is perfected by the produced sebum.

On top of these a number of bioactive molecules, the so-called anti-microbial peptides (AMP) and lipids (AML) are also produced by the cells of the skin. These molecules are crucially important in the organization of the further levels of the protection, i.e. in the formation of the immunological and the closely related microbiological barriers.

Composition of the microbe communities of our skin show characteristic inter- and intraindividual (i.e. region-dependent) variations. Instead of the previously widely accepted "commensal" model, in light of the discoveries of the recent years, it is now thought that a rather dynamic, self-regulating, symbiotic model can better describe the interactions of the skin and its microbiota, where interactions between the skin cells and the microbes can be understood as a special "dialogue". One participant of this "dialogue" is the immunological barrier.

It is well-known now that the skin immune system is not restricted to the resident and immigrating "professional" immune cells, since, due to the expression of pathogen-associated molecular pattern recognizing receptors,

inflammatory cytokines and chemokines, AMPs and AMLs, the epidermal keratinocytes as well as the cells of the sebaceous glands (i.e. the sebocytes) play a key role in it.

The atopic dermatitis (AD)

Atopic dermatitis (AD) shows particularly high prevalence in industrial countries, and can be characterized by chronic cutaneous inflammation. This disease is very often considered "the" barrier disease, because in this case impairment of all components - physicochemical, immunological and microbiological - of the CEB can be observed.

Importantly, some aspects of its pathogenesis are still unclear. According to some researchers, the initial step is the disturbance of the physicochemical barrier due to e.g. mutations of the filaggrin gene. This may enable the colonization and penetration of pathogenic and facultative pathogenic microbes into the deeper layers of the skin, where they can induce a characteristic, T helper 2 (Th_2)-polarized immune response. This, most probably via the produced cytokines, could contribute to the further disorganization of the physicochemical barrier, whereas others claim the dysregulation of the skin immune system to be the primary cause. Although the answer to this question is not yet clear, it is obvious that epidermal keratinocytes are key players of the process in both cases. On one hand, physiological functions of the keratinocytes are indispensable for the formation of the physicochemical barrier; whereas on the other hand, cytokines produced by the keratinocytes are very important in the Th_2-polarization of the AD-accompanying immune response, and the development of the excessive itching, which (due to the increased scratching) can further contribute to the impairment of the physicochemical barrier.

All in all, acquiring a deeper knowledge about the immunological functions of keratinocytes, together with the exploration of the signaling systems regulating it, hold out the promise to provide better understanding of the pathogenesis of AD and by doing this, it augurs the hope of the development of

novel, causative therapeutic tools. Therefore, in the keratinocyte-focused part of the current study we aimed at exploring the function and effects of such a regulator, i.e. the so-called “endocannabinoid system” (further information about it can be found in the corresponding chapter).

Biology of the human sebaceous glands (SG)

The most important role of the SGs is the production of the highly species-specific, neutral lipid-rich sebum. SGs are very important in protecting the skin starting from intrauterine life, and also in the postnatal period, when they are responsible for the three dimensional distribution of the lipids of the skin surface, contributing thereby to the maintenance of the integrity of the physicochemical barrier. They are also important in photo-protection, thermoregulation as well as in transporting anti-oxidants. They influence hair growth, and (by expressing enzymes involved in steroidogenesis and producing androgen hormones) serve as autonomic endocrine organs and stem cell reservoirs. Furthermore sebocytes express Toll-like receptors (TLR), pro-inflammatory cytokines (e.g. interleukin [IL]-6, IL-8, tumor necrosis factor-α etc.), AMPs and AMLs, thus they can be considered immunocompetent cells.

Acne

Acne (a.k.a. “zits”, “pimples”) is one of the highest prevalence human skin diseases, affecting almost everyone for shorter or longer periods. Although not being a directly life threatening disease, its more severe forms and/or those localized to the exposed skin surfaces, can lead to a substantial psychological burden resulting in serious secondary disorders (anxiety, depression or in the worst case suicidal attempts).

The etiology of acne is not fully elucidated yet. However, it is widely accepted that (i) increased and qualitatively altered sebum production (seborrhea), (ii) infundibular hyperkeratosis leading to the closure of the

sebaceous canal and (iii) inflammation evoked by e.g. *Propionibacterium acnes* strains colonizing the stagnating sebum are important parts of the pathogenesis.

Among the various available therapeutic possibilities isotretinoin can be considered the most efficient anti-acne medication because of its efficiency and versatility (it reduces lipogenesis, inhibits infundibular hyperkeratinization, alleviates inflammation and also suppresses the growth of *P. acnes*). Unfortunately, however, development of several, in some cases very serious side-effects (e.g. anemia, neutropenia, thrombocytopenia, liver damage, teratogenicity, psychological problems etc.) should also be taken into account in course of its administration, especially in case of systemic application. Thus, it is easy to understand that development of novel therapeutic tools targeting as many pathological aspects of acne as possible, while exerting a more "favorable" side-effect profile as compared to the already available drugs is a key challenge of the current acne- and SG-focused research efforts.

The model of the human SGs: the SZ95 human sebocyte cell line

Exploration of the physiology of the human SGs was hindered by the lack of reliable animal models representing the whole complexity of the disease. Moreover, isolation and culture of primary human sebocytes turned out to be a great challenge too, since primary sebocytes cannot be cultured for more than a few passages.

Thus, in light of all these, in our current study we employed the best characterized cellular model system, i.e. the SZ95 sebocytes. Moreover, in order to further increase the *in vivo* and clinical relevance of our results, we complemented our experiments by using the full-thickness human skin organ culture (hSOC) technique, which, in the aforementioned regrettable lack of appropriate animal models, is the most "*in vivo*-like" model system at the level of the pre-clinical investigations.

The (-)-cannabidiol (CBD)

It is well-known that "consumption" of the plant *Cannabis sativa* is usually accompanied by characteristic physiological and psychological effects. These are evoked by the so-called "phyto-", i.e. plant-derived cannabinoids (pCB). To date, the number of the identified pCBs exceeds one hundred. Importantly, in contrast to (-)-*trans*-Δ^9-tetrahydrocannabinol (THC), the vast majority of these pCBs do not evoke any psychotropic effects. The best studied non-psychotropic pCB is (-)-cannabidiol (CBD), which is already in use in the neurological clinical practice (in a fixed-dose combination with THC) without any severe side-effects.

Beneficial effects of CBD (e.g. analgesic, anti-inflammatory, anxiolytic, spasmolytic, etc.) are known to be mediated via a great variety of molecular targets. In recent years, it has been shown to activate multiple transient receptor potential (TRP) channels, 5-HT_{1A} serotonin and A_{2A} adenosine receptors, and to antagonize (among others) GPR55, TRPM8, 5-HT_3 and μ opioid receptors. Moreover, it is also able to inhibit several key pro-inflammatory enzymes (cyclooxygenases and lipoxygenases), and to increase the activity of phospholipase A2. Interestingly, the literature is contradictory about its actions exerted on the "classical" CB_1 and CB_2 cannabinoid receptors.

The endocannabinoid system (ECS)

The ECS is comprised of receptors sensitive to different cannabinoids, their endogenous ligands, the so-called "endocannabinoids" (eCB) and the enzyme and transporter apparatus responsible for the synthesis and degradation of the latter. Besides the best known eCBs (anandamide [AEA] and 2-arachidonylglyecrol), several new endogenous substances turned out to be capable to activate cannabinoid receptors recently. Investigation of cannabinoid signaling is difficult not only because of the variation of the ligands, but also because of the presence of a wide-array of metabotropic (e.g. CB_1, CB_2, GPR55,

etc.), ionotropic (certain TRP channels) as well as intranuclear (peroxisome proliferator-activated receptors [PPAR]) receptors that have been proven to be activated or antagonized by various endo- and pCBs. This "pharmacological promiscuity" makes it easier to understand why biological actions of endo- and pCBs can usually be explained by influencing the activity of a characteristic set of target molecules.

The most important and the best characterized actions of the ECS are related to the central nervous system (CNS) and to the immune system, where it was shown to regulate the appetite, memory and mood, and also proven to exert strong anti-inflammatory actions, respectively.

Maybe the most important factor in the development of these effects is the level of the "eCB-tone", which is determined by the activity of the synthesizing and degrading enzymes regulating the amount of the currently available eCBs in the cellular microenvironment. To date, the AEA-degrading fatty acid amide hydrolase (FAAH) appears to be the most important such enzyme.

The cannabinoids and the skin

Various members of the ECS have already been shown to be expressed in (human) skin as well. Among others, it has already been proven that increase of the eCB-tone exerts significant analgesic and anti-pruritic activities. Moreover, the ECS is known to exert complex (i.e. anti-proliferative, pro-apoptotic and anti-angiogenic) anti-tumor activity, and to take part in the regulation of melanogenesis, keratinocyte proliferation and differentiation, regeneration of the CEB, fibroblast functions and the production of the basement membrane, among others.

Role of the ECS in cutaneous inflammation

It is well-known that the ECS exerts highly significant anti-inflammatory activity in the skin. Indeed, disturbed eCB signaling of the epidermal keratinocytes was shown to increase pro-inflammatory chemokine release,

which, via recruiting professional immune cells, initiated and maintained allergic dermatitis. On the other hand, specific knock out of FAAH expression and the subsequent increase in the eCB-tone was efficient in alleviating the symptoms of 2,4-dinitrofluorobenzene-induced dermatitis in mice.

These data, together with several other findings obtained in various mouse models, collectively indicate that the homeostatic eCB-tone developed by the epidermal keratinocytes plays a crucial role in restricting inflammatory processes. As discussed before, FAAH is central negative regulator of this tone. Thus, it is safe to assume that its enhanced expression and activity may contribute to the development of inflammation; whereas restoration of the "homeostatic" FAAH activity might be able to prevent such effects. Therefore, taking into consideration all these, in the course of our ECS-focused experiments we aimed at exploring the putative role of FAAH in the inflammatory processes of human keratinocytes.

ECS and skin appendages

We have recently shown that some members of the ECS are also expressed in the cells of several skin appendages, profoundly influencing their biological functions. From the point-of-view of the current study, the most important piece of these results is that the ECS, besides acting on hair follicles and sweat glands, also affects the functions of the SGs. According to our results, sebocytes are both sources and targets of the eCBs, which, most probably via autocrine and paracrine regulation, play an important role in the maintenance of the basal sebaceous lipid production. We could also show that selective gene silencing of CB_2 (i.e. the abrogation of the autocrine/paracrine "pro-lipogenic" loop) significantly decreased the lipogenesis of SZ95 sebocytes. On the other hand, eCB-treatment of the cells was found to dramatically increase the synthesis of the neutral lipids via activating the CB_2 receptor → ERK1/2 MAPK → PPARγ pathway, raising the possibility that eCB-dysregulation might play a role in the development of acne.

Although these results have unambiguously confirmed that the ECS plays an important regulatory role in human SGs, there were no data available about the putative effects of the pCBs; thus, taking into consideration the aforementioned findings, the other direction of our investigations aimed to uncover the effects of the pCBs (starting with CBD) on human SGs.

AIMS

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

1. How does the non-psychotropic pCB CBD affect the biological functions of human sebocytes, and what signaling pathway(s) may mediate these putative effects?

2. What is the role of FAAH in regulating the pro-inflammatory processes of human epidermal keratinocytes?

MATERIALS AND METHODS

Cell culturing

Human SG-derived immortalized 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, 50 IU/ml penicillin and 50 μg/ml streptomycin. In those cases when we performed our experiments in "low Ca^{2+} medium" in order to clarify the role of extracellular Ca^{2+}, we omitted the aforementioned Ca^{2+}-supplementation.

Human Papilloma Virus E6 antigen-transfected (and thereby immortalized; HPV-KER) and primary human epidermal keratinocytes (NHEK) were cultured in serum-free EpiLife medium, supplemented with 1 (V/V)% Human Keratinocyte Growth medium, 1 (V/V)% antibiotics (pre-formed mixture of penicillin and streptomycin) and 0.5 (V/V)% antimycotic solution.

Human skin samples were obtained from patients having no obvious dermatological disorders, who, before undergoing surgical intervention, provided written informed consent to the use of their skin samples for research purposes. Skin sampling, as well as the use of the tissues and cells was approved by the Regional and Institutional Ethics Committee of the University of Debrecen and the Government Office for Hajdú-Bihar County (*protocol number: DE OEC RKEB/IKEB 3724-2012; document number: IX-R-052/01396-2/2012)*. The study adhered to the Helsinki guidelines in all aspects, applying its principles in the daily practice. Isolation and culture of the NHEKs was performed by our technical assistants *Erika Hollósi*, *Lilla Furin* and *Renáta Uzonyi*.

Cells were cultivated at 37°C in humidified, 5% CO_2 containing atmosphere. The medium was changed every other day, and cells were sub-cultured at 70-80% confluence in order to prevent their confluence-induced differentiation.

In order to exclude the non-specific actions of the vehicles in course of the experiments, 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.

Determination of the intracellular lipid content

Investigation of the lipid content was first performed by using light microscopy and Oil Red O staining; then, for semiquantitative analysis, we applied fluorescent Nile Red staining. In the latter case, signals were detected by using FlexStation™ II^{384} or FlexStation 3 devices. Lipidome of the sebocytes was assessed by our Italian collaborative partners (*Emanuela Camera*, *Matteo Ludovici* and *Mauro Picardo*), using rapid resolution reverse phase high performance liquid chromatography (RR-RP-HPLC) and time of flight mass spectrometry (ToF-MS) following their previously optimized protocol.

Assessment of viability

Viability was assessed by using *colorimetric MTT-assay*. Purple formazan crystals formed during the process were dissolved in 10 (V/V)% Triton-X 100-containing mixture of HCl and 2-propanol solution. The absorbance of the resulting solution was then determined using the aforementioned FlexStation 3 instrument at 565 nm. In case of HPV-KERs, TC_{50} values (i.e. concentration related to the 50% of the control signal intensity) was determined by *Origin Pro Plus 6.0* software using "*Exponential Decay 1*" curve fitting function.

Assessment of apoptotic and necrotic processes

To investigate the putative apoptotic processes, we measured the mitochondrial membrane potential fluorimetrically by using *MitoProbe™ $DilC_1(5)$ Assay Kit* (decreased signal intensity indicated apoptosis), whereas necrotic processes were monitored by *SYTOX Green dye* indicating disintegration of the membranes (increase in the signal intensity marked

necrosis). Fluorescent signals were detected by using FlexStation™ II^{384} or FlexStation 3 devices.

Assessment of the proliferation

Proliferation was investigated by using *CyQUANT proliferation assay* monitoring the DNA-content. Signal intensity was detected by using FlexStation 3.

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

Q-PCR measurements were run on an ABI Prism 7000 sequence detection system or on a Stratagene Mx3005P QPCR System using the 5' nuclease assay and TaqMan primers and probes. As internal controls, expressions of glyceraldehide-3-phosphate dehydrogenase, 18S RNA and peptidylprolyl isomerase A were determined.

Whole-genome microarray analysis

Alterations in the gene expression profile upon CBD-treatment including data processing and evaluation (i.e. bioinformatics, such as *Gene Set Enrichment* [GSEA] and *Biological Networks Gene Ontology* [BiNGO] analyses) as well as determination of the genes exhibiting biologically relevant expressional alterations were performed by *ChromoMed Ltd.* (Budapest, Hungary) and A*biomics Ltd.* (Budapest, Hungary; http://www.abiomics.eu).

Experiments were run on three independent sets of control and CBD-treated (10 μM; 24h) samples by using Human Whole Genome Oligo Microarray® (44K) (Agilent Technologies). Expressional alterations were considered to be biologically relevant in case of at least 2-fold, equidirectional alterations in all cases and if global, corrected *P* value was less than 0.05.

Data of the microarray analyses were deposited in the "*Gene Expression Omnibus*" public database of the *National Center for Biotechnology Information*

(NCBI), and are freely accessible via the "*GSE57571*" accession number (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE57571).

Immunofluorescent labeling (IF)

Sebocytes, seeded onto sterile coverslips, were fixed with acetone at room temperature, and were incubated by using rabbit-anti-human primary antibodies. As secondary antibodies, Alexa Fluor® 488-conjugated goat-anti-rabbit antibodies were used. Nuclei were counterstained by using 4',6-diamidino-2-phenilindol (DAPI) nuclear staining. Pictures were taken by using a Nikon Eclipse E600 fluorescent microscope. As negative control, primary antibodies were omitted from the staining protocol.

Western blot

Equal amounts of protein samples were loaded, and sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed. Next, proteins were transferred to nitrocellulose membranes, which were then incubated with primary antibodies diluted in 5 g/100 ml milk containing phosphate buffered saline solution overnight at 4°C. Subsequently horseradish peroxidase-conjugated antibodies were used as secondary antibodies. Signals were visualized by using SuperSignal® West Pico or Femto Chemiluminescent Substrate kits in a KODAK Gel Logic 1500 Imaging System. Densitometric analysis of the bands was performed by *ImageJ 1.49v* software. When applicable, to assess equal loading, membranes were re-probed by using antibodies raised against β-actin or β-tubulin. The optical density of each band was first normalized to the one of the corresponding loading control. Technical execution of ERK1/2, p-ERK1/2, as well as some of the FAAH Western blots was performed by *Lídia Ambrus* and *Renáta Uzonyi*.

Assessment of the cytokine release (ELISA)

In the technical execution of the investigation of the cytokine release, *Lídia Ambrus* and *Judit Szabó-Papp* provided expert contribution. Cells, seeded in a

standardized way, were treated as indicated for 24 hours. Supernatants were then collected, and stored at -80°C until further processing. Finally, amounts of the released IL-6 and IL-8 were determined by using OptEIA kits following the manufacturer's protocol.

In vitro FAAH activity measurements

Activity of FAAH was assessed by our collaborative partners (*Simon Nicolussi* and *Jürg Gertsch*). Enzyme activity was monitored by measuring the hydrolysis of radiolabeled [ethanolamine-1-^{3}H]-AEA following a previously optimized protocol.

Selective gene silencing by using RNA interference technique (RNA$_i$)

SZ95 sebocytes were transfected by using 40 nM small, interfering double stranded RNA oligonucleotides (siRNA) with Lipofectamine® 2000 transfection reagent in OptiMem medium. As a control, cells were transfected by using Stealth RNA$_i$ Negative Control "medium" double stranded siRNA, which does not exhibit homology to any known mRNA sequences ("scrambled" siRNA).

Determination of the intracellular cAMP concentration

SZ95 sebocytes were treated either by CBD (10 μM) or by vehicle (1 [V/V]‰ absolute ethanol) for 1 hour. Then, following the protocol recommended by the manufacturer, cells were lysed in 10^7 cells/ml density, and were investigated by Parameter Cyclic AMP Assay. Results were evaluated by using the on-line accessible application of *MyAssays Ltd.* (http://www.myassays.com/four-parameter-logistic-curve.assay).

Patch-clamp analyses (whole-cell configuration)

Electrophysiological experiments were run by *Balázs Pál*, *Balázs István Tóth* and *Thomas Voets*. To measure transmembrane currents, cells were ramped every 2 seconds over the course of 400 ms, within a wide membrane potential range. Holding potentials were 0 or -60 mV.

Fluorescent Ca^{2+}-measurement

Ca^{2+}-signaling of the sebocytes was investigated in Hank's solution at room temperature (20-22°C) by using Fluo-4 AM dye. Measurements were performed using FlexStation™ II^{384} or FlexStation 3 devices in "Flex" mode. Mean of the pre-treatment background intensity was extracted from the measured values, which were thereby normalized to the same baseline, enabling us to compare our measurements.

Full-thickness human skin organ culture (hSOC)

hSOC experiments were run by our collaborative partners in Lübeck (*Koji Sugawara, Jennifer Kloepper* and *Ralf Paus*). The study was approved by the Institutional Ethics Committee of the University of Lübeck (*reference number: 06-109*) and adhered to the Declaration of Helsinki. Donors provided written informed consent to the use of their skin samples for research purposes. Following the corresponding treatments, determination of the sebaceous lipid production was performed by *Oil Red O staining*, whereas proliferation was assessed by *Ki67 immunolabeling*.

Animal model of AD: experiments on NC/Tnd mice

Experiments were run by employees of *BioTox Sciences* (BTS). Experimental design, as well as animal breeding and welfare was supervised and approved by the Institutional Animal Care and Use Committee (*No. 1109-05*). Experiments and animal welfare were in compliance with the Guide for the Care and Use of Laboratory Animals and BTS SOPs, and adopted the principles of "*Guide for the Care and Use of Laboratory Animals, 8th Edition National Academy Press, Washington, DC, 2010*" guidelines. In the study, 8-9 week old male NC/Tnd (previously known as NC/Nga) mice were investigated (8-9 mice per group). The study was run in two equal halves. These mice are symptom-free while being kept under specific pathogen-free environment. However, upon being challenged by common allergens, an itching dermatitis develops, which

exhibits clinical and histological similarities to human AD. To evoke AD-like symptoms, standardized dust mite antigen extract (15,000 AU/ml *Dermatophagoides pteronyssinus* and *D. farinae*) was employed. Animals were then randomized based on their initial total disease score (TDS) so that the average of the starting scores was cca. 1 in each group. TDS was determined from observations of the upper back/lower neck (i.e., the area that received topical treatment) with a scale of 0–3 (0: absent; 1: mild; 2: moderate; 3: severe) for erythema, edema or papulations, and for oozing, crusts or hemorrhages. TDS and ear thickness were assessed twice a week at the indicated time-points.

Statistical analysis

Data were analyzed by *IBM SPSS Statistics 19* or *Origin Pro Plus 6.0* softwares using Student's paired (patch-clamp analysis) and two-tailed, two samples *t*-tests (paired comparisons) or one-way ANOVA followed by Bonferroni and Dunnett *post hoc* tests (multiple comparisons), and $P<0.05$ values were regarded as significant differences. Homogeneity of variances were analyzed by Levene's test. If it indicated inhomogeneity of variances, Games-Howel test was used instead of Bonferroni. Graphs were plotted by using *Origin Pro Plus 6.0* software.

RESULTS

CBD exhibits complex anti-acne effects both in vitro and ex vivo

First, we aimed at exploring how CBD influences lipid synthesis of the cells. We found that, in contrast to the eCBs, CBD had no effect on the basal lipid production of the sebocytes (*Oil Red O* and *Nile Red staining*). Interestingly, however, it was able to dose-dependently and completely prevent pro-lipogenic effects of not only the eCB AEA, but also other "pro-acne" agents (arachidonic acid [AA] and the combination of linoleic acid and testosterone) which act through ECS-independent lipogenic pathways. These findings highlighted that the lipostatic effect of CBD applied at 1-10 μM may develop not because of the activation of a cannabinoid system-specific, but rather a universal lipid synthesis suppressing signaling pathway (*Nile Red staining*). Furthermore, we could also demonstrate that CBD applied at 1-100 nM had no effect on either the basal or the AA-induced lipid production (*Nile Red staining*), excluding the possibility of a biphasic biological activity kinetics in the case of the lipid synthesis of the sebocytes, which might have been expected based on the data of the literature. Finally, by investigating the sebocyte lipidome, we showed that CBD not only quantitatively, but also qualitatively normalized the AA-induced, acne- and seborrhea-mimicking sebaceous lipid overproduction (*RR-RP-HPLC-ToF/MS analysis*).

Altogether, these data suggested that CBD might well be an efficient anti-acne agent. Thus, in the hope of exploring its further putative beneficial anti-acne effects, we found that CBD dose-dependently reduced proliferation of the sebocytes when applied at 1-10 μM (*CyQUANT-assay*). By using *MTT-assay* and combined *DilC$_1$(5)-SYTOX Green labeling*, we could also show that the aforementioned cell count reducing activity was not mediated by cytotoxic effects, but rather by "pure" anti-proliferative actions. On the other hand, when

administered at high concentration (50 μM) or for longer period of time (10 μM for 6 days), it induced apoptosis and suppressed sebaceous lipid production.

Our data suggested therefore that in course of CBD administration, one might be able to define such concentration-duration combinations in which full-fledged sebostatic (i.e. simultaneous lipostatic and anti-proliferative) actions can develop; without suppressing either basal sebaceous lipid synthesis or viability of the sebocytes. All this pointed towards a highly targeted therapeutic possibility in treating acne, i.e. to selectively treat the acne lesions without compromising fundamental functions of the non-lesional SGs. Obviously, from the point-of-view of the hoped side-effect-free therapy, clinical significance of such findings may be very high.

Next, in order to increase the *in vivo* relevance of our findings, with the lack of appropriate animal models, we also assessed the sebostatic effect of CBD by employing hSOC technique. We found that CBD efficiently decreased the AEA-induced lipid production, and also suppressed the basal lipid synthesis over the course of long-term experiments (*Oil Red O staining*). Similarly, CBD was efficient in reducing the ratio of proliferating (i.e. Ki67 positive) sebocytes. These findings suggested that beneficial *in vitro* anti-acne effects of CBD are highly likely to develop *in vivo* as well.

Besides the quantitatively increased and qualitatively altered sebum production, pathogenesis of acne consists another important "sebocyte-specific" step: local inflammation. Therefore, a potent anti-acne agent is highly desirable to exert not only sebostatic, but also anti-inflammatory actions. In order to assess this, as the last step of our phenomenological experiments, we employed different inflammation models in human sebocytes. We found that co-administration of CBD had normalized lipopolysaccharide (LPS), lipoteichoic acid (LTA) as well as linoleic acid+testosterone combination-evoked elevations in the expressions of certain pro-inflammatory cytokines (*Q-PCR*), i.e. CBD had been shown to exert universal anti-inflammatory actions.

Lipostatic and anti-proliferative actions of CBD are mediated by TRPV4

Having demonstrated that lipostatic actions of CBD develop independently of the metabotropic CB_1 and CB_2 cannabinoid receptors (*Nile Red staining*), we started the systematic exploration of further potential targets. Using whole-cell configuration of conventional patch-clamp technique we demonstrated that CBD applied at 10 μM induced outwardly rectifying currents, and shifted the reversal potential towards more positive values. These findings suggested that administration of CBD might lead to the opening of surface membrane cation channels on the sebocytes, directing our attention towards the ionotropic CBD-sensitive receptors, i.e. the TRP channels. We have previously shown that activation of one of these channels, i.e. TRPV1 by capsaicin resulted in a CBD-like lipostatic activity. Thus, next we investigated if TRP channels might play any role in mediating the effects of CBD.

We found that TRPV1, -2 and -4 ionotropic cannabinoid receptors are expressed in human sebocytes both at the mRNA (*Q-PCR*) and protein (*IF*, *Western blot*) levels. Moreover, we could also demonstrate that TRPV4 is expressed in a functionally active form (*patch-clamp*, *Fluo-4 AM-based fluorescent Ca^{2+}-measurement*), and that CBD-induced increase of the intracellular Ca^{2+}-concentration is mediated via this channel (*Fluo-4 AM-based fluorescent Ca^{2+}-measurement*). Next, by employing pharmacological receptor modulators and RNA_i technique we showed that the lipostatic activity of CBD is exclusively mediated by TRPV4, whereas TRPV1 and -2 do not play any role in the process (*Nile Red staining*). Finally, in course of the investigation of the remaining anti-acne modalities, we found that the anti-proliferative effect is also TRPV4-dependent (*CyQUANT-assay*), whereas the anti-inflammatory action develops in a TRPV4-independent manner (*Q-PCR*).

CBD's sebostatic effect is mediated via the TRPV4-dependent inhibition of the ERK1/2 MAPK pathway and via the also TRPV4-coupled down-regulation of nuclear receptor interacting protein 1 (NRIP1)

Having excluded the participation of several important intracellular regulatory molecules (PKC isoforms, PI3K, PKA, calcineurin) in mediating the lipid synthesis reducing effects, we continued our experiments by *whole-genome microarray analyses*. Upon studying three independent sets of control and CBD-treated (10 μM, 24 hours) sebocytes, *GSEA* and *BiNGO* analyses clearly confirmed the development of complex anti-acne actions. Moreover, we also found that expression of 80 genes was significantly down-, whereas of 72 was significantly up-regulated upon the aforementioned CBD-treatment. By using *Q-PCR*, we could confirm that expressions of nuclear receptor interacting protein 1 (NRIP1; positive regulator of the triglyceride storage of the adipocytes) and Ki67 proliferation marker were indeed down-regulated in a TRPV4-dependent manner, whereas expression of Rho GTPase activating protein 9 (ARHGAP9; endogenous inhibitor of the pro-lipogenic ERK pathway) was (again, TRPV4-dependently) increased. We could also demonstrate that CBD inhibited AEA-induced activation of the ERK1/2 MAPK pathway (*Western blot*) in a TRPV4-dependent manner, and that RNA_i-mediated knock down of NRIP1 (mimicking the cellular effects of CBD) decreased AA-induced lipogenesis of sebocytes in a CBD-like way (*Nile Red staining*).

Anti-inflammatory action of CBD develops most probably via tribbles homolog 3 (TRIB3) mediated inhibition of the P65-NF-κB pathway

Following in-depth exploration of the cellular processes responsible for the development of the sebostatic activity, we intended to unveil the details of the anti-inflammatory pathway. Q-PCR validation of the aforementioned microarray data revealed that, in line with our previous findings, CBD up-regulated the expression of several genes, which are known to affect immune functions (i.e. tribbles homolog 3 [TRIB3], an inhibitor of the NF-κB pathway and the AMP

LL-37 cathelicidin) in a TRPV4-independent manner. We could also demonstrate that selective gene silencing of TRIB3 prevented the anti-inflammatory actions of CBD (*RNA_i, Q-PCR*), but it had no effect on the lipostatic activity (*Nile Red staining*). Bearing in mind that TRIB3 is a known endogenous inhibitor of the P65-NF-κB pathway, we also investigated how CBD influences the LPS-induced activation of the NF-κB pathway. We found that, in line with the data of the literature, and as well as with our aforementioned results, CBD was able to prevent LPS-induced phosphorylation (and thus inactivation) of the inhibitory I-κB-α and phosphorylation (and hence activation) of P65-NF-κB (*Western blot*). Collectively, these findings suggest that anti-inflammatory actions of CBD are mediated via the (most probably TRIB3-mediated) inhibition of the NF-κB pathway.

CBD exerts its anti-inflammatory action via activating the A_{2A} adenosine receptor

When exploring the up-stream signaling of the TRIB3 up-regulation, we found that CBD increases the intracellular cAMP level of the sebocytes (*ELISA*). Knowing that the A_{2A} adenosine receptor is the single known G_s-protein-coupled molecule among the possible targets of the CBD, we investigated its expression using *Q-PCR*, *Western blot* and *IF labeling*. We found that the A_{2A} receptor is indeed expressed by human sebocytes both at the mRNA and protein levels. Moreover, its inhibition was able to equally prevent (i) CBD-induced up-regulation of TRIB3 (*Q-PCR*); (ii) development of the anti-inflammatory effects (*Q-PCR*); and (iii) abrogation of the activation of the NF-κB pathway (*Western blot*).

All in all, our results indicate that, in human sebocytes, anti-inflammatory actions of CBD are mediated via the A_{2A} receptor → cAMP↑ → TRIB3↑-mediated inhibition of the P65-NF-κB pathway.

Activation of Toll-like receptor 2 (TLR2) increases expression and activity of FAAH in human epidermal keratinocytes

In the second part of our study, we aimed to achieve better insight into the role of the ECS in regulating pro-inflammatory processes of epidermal keratinocytes.

First, we successfully reproduced data from the literature claiming that FAAH is functionally expressed in human epidermal keratinocytes. It is well-known that the FAAH-regulated eCB-tone exerts a continuous, "homeostatic" anti-inflammatory "pressure" on the keratinocytes. On the basis of this, one can assume that in course of the initiation of local inflammatory processes, FAAH-mediated negative regulation of the eCB-tone might contribute to the fine-tuning of the magnitude of the biological response. Indeed, in line with this hypothesis, we found that activation of TLR2 significantly increased protein (but, interestingly, not mRNA; *Q-PCR*) level expression as well as activity of FAAH (*Western blot*; *FAAH activity measurement*) in epidermal keratinocytes.

Inhibition of FAAH results in significant anti-inflammatory actions via the indirect activation of CB_1 and CB_2 receptors

To further dissect the (patho)physiological role of FAAH, we evoked pro-inflammatory responses on epidermal keratinocytes by activating TLR2 with LTA. The response was monitored by measuring the expression (*Q-PCR*) and release (*ELISA*) of various pro-inflammatory cytokines (mRNA level: IL-1α, IL-1β, IL-6 and IL-8; protein level: IL-6 and IL-8). Using selective commercially available (URB597), as well as novel FAAH inhibitors developed by our collaborative partner (WOBE440 and WOBE479) we found that inhibition of FAAH efficiently suppresses the LTA-induced *in vitro* inflammatory response (*Q-PCR*, *ELISA*). Moreover, by employing selective CB_1 and CB_2 receptor antagonists, we could also demonstrate that this action was mediated via the

elevation of the eCB-tone leading to the (most probably indirect) activation of these receptors (*Q-PCR*).

FAAH inhibitors can be applied without the risk of cytotoxicity

Next, we aimed to assess the effects of the FAAH inhibitors on the cells' viability. We found that upon 8- or 24-hr treatments none of the inhibitors influenced viability of HPV-KERs (*MTT-assay*) or induced initiation of early apoptotic processes (*DilC_1(5)-SYTOX Green labeling*). Repetition of our experiments by using long-term (i.e. 72-hr) treatments further confirmed the above statement. We found that cytotoxicity only developed when the inhibitors were administered in concentrations several orders of magnitude above their efficient anti-inflammatory concentrations (*MTT-assay*).

FAAH inhibitors alleviate cutaneous symptoms of NC/Tnd mice

Last, but not least, we also intended to know, whether FAAH inhibitors are able to exert their beneficial actions in a clinically relevant *in vivo* model system, following topical administration.

To answer this question, our collaborative partners investigated the efficiency of FAAH inhibitors in treating cutaneous symptoms of NC/Tnd (previously known as NC/Nga) mice. As "positive" control, a calcineurin inhibitor (tacrolimus) was used. It has been shown that topical application of the two, newly synthesized FAAH-inhibitors was proven to be practically "tacrolimus-equivalent" in alleviating skin symptoms (TDS), and in suppressing ear swelling, a widely accepted, general marker of the inflammatory processes. Moreover, over the course of the 30-day drug administration period neither macroscopically obvious side-effects, nor behavioral alterations were revealed. Altogether, these findings indicate that topically applied FAAH inhibitors may also exhibit *in vivo* efficiency in the clinical treatment of various cutaneous inflammatory diseases.

DISCUSSION

Better understanding of cutaneous (patho)physiology holds out the promise of developing novel therapeutic approaches in the case of several high-prevalence diseases. The ECS and (endo)cannabinoid signaling are a recently discovered, intensively "growing" regulatory system, which (based on the data of the literature) are very likely to play a central role in fine-tuning immune responses. Thus, their dysregulation may also contribute to the development of several common, inflammation-accompanied skin diseases (e.g. AD, acne, etc.). As such it may well be a key future target of molecular medicine. Therefore, within the confines of the current work, we intended to investigate the effects of a non-psychotropic pCB, CBD, on human sebocytes, and to study the functional significance of one of the most important eCB-degrading enzymes, i.e. FAAH, from the point-of-view of its effects on the immunological properties of human epidermal keratinocytes.

FAAH as a putative target in the management of dermatitis

It is well-known that, by releasing various chemokines and cytokines, epidermal keratinocytes are key players in recruiting "professional" immune cells as well as in regulating their functions. The biological activity of keratinocytes therefore plays an important role in the initiation and regulation of cutaneous inflammatory processes. Although epidermal expression of FAAH has already been described, we demonstrated for the first time that its activity (but interestingly, not its mRNA expression) was significantly increased following 24-hr treatments by the TLR2-activating LTA both in immortalized as well as primary human epidermal keratinocytes. Thus, posttranscriptional regulation of the enzyme activity (most probably via decreasing the homeostatic eCB-tone) appears to be able to contribute to the perfection of inflammatory processes.

In light of these results, normalization of the TLR2-induced FAAH activity held out the promise of a brand-new, targeted anti-inflammatory therapeutic tool in managing cutaneous inflammatory processes. In order to challenge this hypothesis, we investigated how a commercially available (URB597) and two potent, selective and newly developed (by our collaborative partners) FAAH inhibitors (WOBE440 and WOBE479) influence the development of the LTA-induced inflammatory response. We found that the aforementioned inhibitors significantly suppressed the effect of LTA. We could also demonstrate that, in line with data found in the literature, indirect activation of the CB_1 and CB_2 cannabinoid receptors underlay the effects. These data obtained by investigating keratinocytes, suggested that FAAH inhibitors may be able to exert efficient anti-inflammatory actions *in vivo* too. Moreover, according to certain theoretical considerations, this effect may be further supported by other local factors, since increase of the epidermal eCB-tone should (hypothetically) shift biological behavior of e.g. Langerhans cells as well as of sensory nerve endings towards an anti-inflammatory direction.

From the point-of view of the putative clinical application, it is crucially important whether one prefers to administer a drug systemically or topically. Taking into consideration that in case of topical application one has the chance to execute a more targeted treatment (and hence to use maybe even higher concentrations of the drug), in order to minimize the risk of the development of putative side-effects, direct topical administration appears to be the most expedient solution. Taking into consideration all the above, as the final step of the current study, we also intended to test the anti-inflammatory efficiency of the FAAH inhibitors by using NC/Tnd mice, a widely accepted animal model of AD.

We found that the topically administered FAAH inhibitors suppressed ear swelling, a general marker of the inflammatory processes, and significantly reduced TDS as well. Moreover, without inducing any obvious macroscopic or

behavioral side-effects over the course of the one-month long examination period, their efficiency was comparable to that of tacrolimus, which was applied as a "positive" control. Thus, results of our currently presented, complementary *in vitro* and *in vivo* experiments indicate that – at least according to the animal study –inhibition of FAAH may become an "*in vivo* side-effect-free" therapeutic tool in the future treatment of various dermatites.

Complex anti-acne effects of CBD and their mechanisms

Acne is one of the most common human skin diseases. Its treatment is still not fully satisfactorily solved. In our current study, by using human, immortalized SZ95 sebocytes as well as hSOC, we demonstrated that CBD, a pCB, being already in use in the adjuvant therapy of sclerosis multiplex, exerts complex anti-acne effects. Indeed, according to our results, CBD, without compromising either the viability or basal sebaceous lipid synthesis of sebocytes, was able to normalize the pro-acne agent-induced elevated lipid production, both in a quantitative and qualitative manner, and it also exhibited anti-proliferative and anti-inflammatory actions. Moreover, its sebostatic action also developed under "*in vivo*-like" circumstances in hSOC.

Besides the aforementioned triple cellular anti-acne activity, according to data in the literature, topical administration of CBD may be followed by the development of further highly desirable effects. Indeed, CBD has recently been proven to possess substantial anti-bacterial activity, and, although its putative efficiency against *P. acnes* has not been tested yet, it highlights the possibility that its indirect (i.e. mediated by the up-regulation of LL-37 cathelicidin) anti-bacterial activity may be accompanied by direct ones. Moreover, CBD was also shown to suppress proliferation and differentiation of hyperproliferative keratinocytes. This, together with the normalization of the sebaceous lipidome and the suppressed expression of certain pro-inflammatory cytokines may beneficially affect comedogenesis. Thus, theoretically, upon CBD

administration, a complex, global anti-acne profile can develop covering all major pathogenetic steps of acne at the same time.

Having the phenomenological characterization of CBD's beneficial effects completed, we next intended to unveil the details of their mechanism(s) of action. Taking into consideration the large number of the putative target molecules of CBD, this augured to be a very challenging task. Importantly, some of our previous results (i.e. activation of TRPV1 leads to the development of CBD-like lipostatic actions) already raised the possibility that activation of ionotropic cannabinoid receptors may play a role in mediating CBD's effects as well. In line with this hypothesis, using a wide array of electrophysiological, pharmacological and molecular biological methods, we have shown that CBD's lipostatic and anti-proliferative activities are coupled to the activation of TRPV4 ion channels and the subsequent Ca^{2+}-influx, whereas the anti-inflammatory action was proven to be TRPV4-independent.

At this point, it is also noteworthy that negative regulation of the lipid synthesis by Ca^{2+}-coupled signaling is not unprecedented in the literature, since besides the sebocytes, similar mechanisms were already described in adipocytes as well. Moreover, our current findings are perfectly in line with the ones of other groups, who already reported that CBD was a potent, but, as compared to the "classical" agonists or to some other pCBs (e.g. (-)-tetrahydrocannabivarin [THCV]) less efficacious activator of TRPV4. Importantly, according to our yet unpublished observations, THCV, which is, again, more efficacious activator of TRPV4 as compared to CBD, exerted CBD-like sebostatic actions at the 1-10 μM concentration range, but (unlike CBD) at 10 μM it also suppressed basal sebaceous lipid synthesis and evoked higher Ca^{2+}-signals as compared to the ones being characteristic to CBD.

Importantly, our microarray analyses, performed to get better insight into the mechanisms of the anti-acne effects, highlighted the putative role of several potential target genes, which results could be validated by QPCR. Namely, we

found that genes related to the regulation of the lipid synthesis (NRIP1 and ARHGAP9) and proliferation (Ki67) showed TRPV4-dependent, whereas those ones possessing immunological functions (TRIB3 and LL-37 cathelicidin) exhibited TRPV4-independent regulation.

Based on these results, we could confirm that lipostatic activity develops via the TRPV4-dependent inhibition of the pro-lipogenic ERK1/2 MAPK cascade, and the (also TRPV4-dependent) down-regulation of NRIP1, a positive regulator of triglyceride storage. In contrast, the anti-inflammatory actions appear to be coupled to the inhibition of the pro-inflammatory P65-NF-κB pathway most probably via the A_{2A} adenosine receptor → cAMP↑ → TRIB3↑ cascade.

All in all, our data introduce CBD as a potentially very efficient anti-acne agent. A real multitarget drug; being able to beneficially influence all key events of the pathogenesis in the same time. Of great importance, safety of CBD is reported in many studies, and is also confirmed in clinical practice. This fact makes CBD a very attractive molecule – especially if we take into consideration the significant side-effect profile of the currently available most versatile drug, isotretinoin. Therefore, these data together with our current findings highlight the possibility of a cost-effective and most probably well-tolerated, but still very efficient CBD-based acne therapy.

Although the pharmacokinetics of CBD in the human body is not fully uncovered yet, based on its high lipophilicity, it is safe to assume that it would accumulate in the lipid-rich SGs, maybe reaching relatively high, even therapeutically sufficient concentrations in the case of systemic administration. On the other hand, as compared to the systemic one, its targeted topical administration augurs to be an even more promising possibility. This appears to be a particularly intriguing possibility, because in this case, due to its high lipophilicity, CBD is known to penetrate into the deeper skin layers via the

transfollicular route, making it highly likely that it would accumulate in the originally targeted SGs.

Although the efficiency of "pure" CBD for the treatment of seborrhea or acne has not been investigated yet in appropriately controlled clinical trials, one recently published paper suggests the possible clinical efficiency of CBD. In this article, the Pakistani authors presented the results of a 12-week long, placebo-controlled, single-blinded clinical trial, in which, 3% hempseed extract, or vehicle-containing cream was tested in a split-face study. Importantly, they found that the *Cannabis* extract significantly decreased sebum production and erythema as compared to the vehicle-treated half.

Collectively, according to our aforementioned results, CBD (especially in light of the available data in the literature, see above) appears to be an extremely promising and efficient anti-acne agent possessing a favorable side-effect profile. Therefore clinical trials are urgently invited to exploit its putative *in vivo* efficiency to decide if it was indeed as efficient as our results together with the data of the literature would suggest. On the other hand, appropriate targeting of the revealed, previously unknown sebostatic (TRPV4 → Ca^{2+}↑ → ERK1/2 MAPK↓ and NRIP1↓) and anti-inflammatory (A_{2A}→cAMP↑→TRIB3↑→P65-NF-κB↓) signaling pathways may open new routes for developing further targeted and highly efficient agents possessing favorable side-effect profiles.

SUMMARY

In the current study we aimed at investigating the effects of a non-psychotropic phytocannabinoid, (-)-cannabidiol (CBD) on human sebocytes, and the fatty acid amide hydrolase (FAAH) enzyme, a key negative regulator of the anti-inflammatory endocannabinoid tone (eCB-tone) on epidermal keratinocytes as well as on NC/Tnd mice.

We found that protein expression and activity of FAAH was increased by Toll-like receptor (TLR)-2 activation both in immortalized and primary human epidermal keratinocytes, indicating that FAAH-mediated decrease of the anti-inflammatory eCB-tone might theoretically contribute to the development of TLR2-induced inflammatory processes. By using various FAAH-inhibitors, we furthermore demonstrated that the consequently increasing eCB-tone exerts substantial anti-inflammatory actions via activating CB_1 and CB_2 receptors both *in vitro* on human keratinocytes and *in vivo* on NC/Tnd mice. Taken together, these results indicate that FAAH-inhibitors may become powerful tools in the treatment of various cutaneous inflammatory disorders.

By administering CBD (a substance in use in neurological clinical practice without any significant side-effects for many years) we could demonstrate that it exerted complex anti-acne activity (combined lipostatic, anti-proliferative and anti-inflammatory actions), which were mediated via novel sebostatic (TRPV4→Ca^{2+}↑→NRIP1↓ and ERK1/2 MAPK↓) and anti-inflammatory (A_{2A}→cAMP↑→TRIB3↑→P65-NF-κB↓) pathways.

Since according to data in the literature CBD is very likely to exert beneficial effects against other relevant pathogenetic steps of acne as well (comedogenesis, colonization of *P. acnes*), our results raised the possibility that CBD may be a powerful tool in the future acne therapy.

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LIST OF PUBLICATIONS

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List of publications related to the dissertation

1. **Oláh, A.**, Ambrus, L., Nicolussi, S., Gertsch, J., Tubak, V., Kemény, L., Soeberdt, M., Abels, C., Bíró, T.: Inhibition of fatty acid amide hydrolase exerts cutaneous anti-inflammatory effects both in vitro and in vivo.
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