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

Investigation of the Novel Aspects of Human Sebaceous Gland Biology: Focus on the Non-Psychotropic Phytocannabinoids and the Nicotinic Acid

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2020

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April 25, 2018, 11:00

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13, 03, 2020, 13:00

Introduction

The focus of our research has been the various diseases of the human skin, and specifically their molecular background. Acne is one of the most common human skin diseases. Although not directly life-threatening, particularly severe forms can greatly impair the patients' quality of life and lead to secondary psychiatric disorders. Given that currently available therapeutic agents (although they are effective in relieving the symptoms of the disease) can cause serious side effects, there is a great unmet need for a deeper understanding of the biology of sebaceous glands (SGs) and the identification of new therapeutic approaches with more favorable side effect profiles.

Our team has previously demonstrated that the endocannabinoid system (ECS) and, more broadly, multifaceted cannabinoid signaling, regulate the biological processes of SGs in a context-dependent, varied manner. Inhibition of endocannabinoid (eCB) degradation as well as direct eCB treatment enhances sebum lipid synthesis, while the non-psychotropic phytocannabinoid (pCB), (-)-cannabidiol (CBD) – which is already used in neurological clinical practice – exhibits complex anti-acne effects. These include quantitative and qualitative normalization of sebum lipid production, anti-proliferative and anti-inflammatory activities *in vitro* (in cell culture), *ex vivo* (in whole thickness human skin organ culture), and *in vivo* (according to the latest clinical studies where CBD was applied in a topical formulation).

Given that there are no data in the literature on the effects of pCBs other than CBD on sebocytes, several non-psychotropic pCBs ((-)-cannabichromene [CBC], (-)-cannabidivarin [CBDV]), (-)-cannabigerol [CBG], (-)-cannabigerovarin [CBGV]

and (-)-Δ^9-tetrahydrocannabivarin [THCV]) were analyzed in human sebocytes, and are reported in the present study.

Nicotinic acid (niacin; NA) and nicotinamide are members of the vitamin B3 complex, the absence of which leads to the development of diarrhea, dementia, dermatitis, and a special type of SG disorder ("dyssebacia"). NA is also effective at treating certain dyslipidemias at pharmacological doses (through activation of the hydroxycarboxylic acid receptor 2 [HCA_2]). Given that side effects of NA at the pharmacological dose include dry skin, whereas NA deficiency may lead to the development of a SG disorder, in the second part of our study we investigated how NA influences the biological processes of sebocytes.

Aims

In the light of the above, we have aimed to answer the following questions in the framework of the studies presented in this dissertation:

1. Do other non-psychotropic pCBs similar to CBD, namely CBC, CBDV, CBG, CBGV and THCV, influence the biological processes of human sebocytes?

2. Does NA affect the basal (homeostatic) and acne-related pathologically increased sebaceous lipid production, cell proliferation, and possibly the immune profile of sebocytes and, if so, does it exert its effects via the HCA_2 receptor?

Materials and Methods

Culturing and treatment of human immortalized SZ95 sebocytes

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% confluency. *Mycoplasma* contamination of our cultures was tested regularly with the MycoAlert™ PLUS *Mycoplasma* Detection Kit, and in each case a negative result was obtained. Cultivation was carried out in humidified atmosphere (5% CO_2) at 37°C. To exclude possible non-specific solvent effects (except for NA directly dissolved in the medium), a stock solution of was prepared and stored at -20°C or 4°C as recommended by the manufacturer. Stock solutions were diluted 1:1000 immediately prior to treatment in cell culture medium, making sure that the treatments being compared always had the same "vehicle background". Cells treated with the vehicle of the materials were used as controls.

Determination of intracellular lipid content of sebocytes

For the semiquantitative assay of 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 and 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).

Determination of cellular viability (MTT assay)

Cells were plated in 96-well plates at 20 000 cells/well, and treated with various concentrations of the appropriate materials. 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 of "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.

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.

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, which permeabilized the cells. Subsequently, 200 µl of CyQUANT

GR stock solution diluted according to the manufacturer's protocol was added to each well and incubated for 5 minutes at room temperature, protected from light. Fluorescence intensity was detected using a FlexStation 3 (excitation wavelength: 480 nm; emission wavelength: 520 nm).

Quantitative real-time polymerase chain reaction (Q-PCR) following reverse transcription

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 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* or *GAPDH* was determined as an internal control.

Immunofluorescent Labeling (IF)

Cells were fixed with 4% paraformaldehyde diluted in PBS in a humid chamber at room temperature (5 min). Blocking of non-specific binding sites was done after washing with PBS in PBS containing 30 mg/ml BSA (bovine serum albumin) for 30 minutes at room temperature in a humidified chamber. The cells were incubated for 4 hours with primary antibody against human HCA_2 produced in rabbit at 37°C. The coverslips were then washed three times in PBS and incubated for 60 minutes at room temperature with Alexa Fluor® 488-conjugated secondary antibody against rabbit immunoglobulin Fc segment. Finally, after washing three times with PBS, cells were mounted using Vectashield mounting medium containing 4',6-diamidino-2-phenylindol (DAPI) as a nuclear stain. Cells were photographed using an Olympus IX81 fluorescence microscope (excitation/emission: 488/519 nm [Alexa Fluor® 488] and 360/460 nm [DAPI]). As a negative control, our coverslips were stained with the

omission of the primary antibody. The mean gray value of the images (detected in the green channel, using the same settings for each image exposure) was determined by *Fiji* software and normalized to the background (i.e., the mean gray value of the cell-free area) when quantified.

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 and 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 released was determined using OptEIA kits according to the manufacturer's protocol.

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% confluency, the medium was replaced with serum-free OptiMem medium and cells were duplexed with specific HCA_2-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 (immunofluorescent labeling) on days 2 and 3 after transfection.

Fluorescent Ca^{2+} measurement

To investigate the effects of NA on Ca^{2+} homeostasis, Fluo-4 AM labeling was used. The sebocytes were plated in 96-well plates at 20 000 cells/well. During the assay, cells were washed once with 100 µl/well Hank's solution supplemented with BSA and probenecid to prevent non-specific carrier-mediated Fluo-4 leakage. After

washing, the cells were loaded with Hank's solution (100 µl/well; final concentration: 1 µM) containing Fluo-4 AM (30 min, 37°C), followed by three additional washing steps (100 µl/well Hank's solution or nominally Ca^{2+}-free Hank's solution with equimolar glucose instead of $CaCl_2$). Finally, 150 µl Hank's solution or nominally Ca^{2+}-free Hank's solution was pipetted to the wells, and cells were incubated for another 30 min at 37°C. The measurement was then performed in Flex mode of a FlexStation 3 plate reader at room temperature (excitation wavelength: 490 nm; emission wavelength: 520 nm).

Statistical analysis

Data were analyzed using *IBM SPSS Statistics 19* and *Origin Pro Plus 6.0* software. Data was evaluated with Student's two-tailed, two-sample 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* software.

Results

pCBs influence the viability of sebocytes in a dose-dependent manner

Our previous findings revealed that human SGs have a functionally active ECS, which plays a role in regulating sebocyte differentiation and thereby lipid production. Further, it has been shown that the use of the non-psychotropic pCB CBD at low micromolar concentrations leads to complex anti-acne effects. Accordingly, at the beginning of our experiments, we sought to determine whether the five pCBs we selected produced similar effects on SZ95 sebocytes as CBD. As the starting point of investigating each drug, we wanted to determine the effective dose range that did not significantly affect cell viability, so we first performed an MTT assay. The five selected pCBs did not significantly reduce cell viability after 48 hours compared to control. In contrast, at higher concentrations (50-100 μM), there was a significant decrease in MTT signal intensity even after 24 hours of treatment. Based on the results of the MTT assay performed in the higher concentration range, the use of pCBs, even at lower concentrations (i.e., ≤10 μM), may induce early cell death processes that do not alter the MTT assay. To resolve this issue, we combined $DilC_1(5)$ and SYTOX Green labeling. We found that none of the selected pCBs increased the proportion of necrotic or apoptotic cells at 10 μM. However, higher concentrations of pCBs (50-100 μM) resulted in apoptosis-dominated cell death, as they all significantly reduced the mitochondrial membrane potential ($DilC_1(5)$) and did not (CBG, CBGV, CBC, and CBDV) or only slightly (THCV) increased the signal intensity of SYTOX Green.

pCBs have varied effects on the sebum lipid production of human sebocytes

We next examined the effect of pCBs on one of the most important biological functions of sebocytes, homeostatic sebaceous lipid production. Not surprisingly,

cytotoxic (≥50 μM) concentrations of pCBs typically significantly reduced basal sebaceous lipid synthesis. In contrast, pCBs used at non-cytotoxic concentrations (≤10 μM) showed intriguing differences in their effects. Based on the effects we classified the five substances into three functionally different ("eCB-like", lipogenic, "CBD-like", i.e., neutral, or lipostatic) groups. According to our results, "eCB-like" CBG and CBGV slightly, but significantly increased sebaceous lipid production. In contrast, CBDV produced a "CBD-like" effect, in that there was no significant change, whereas CBC and THCV significantly reduced it. These results suggested that CBC and THCV may have therapeutic potential in seborrhea and acne, whereas CBG and CBGV may have therapeutic potential in skin dryness, which is why we continued our experiments in this direction.

CBG and CBGV reduce the lipogenic effect of AEA

First, we looked at the surprising, "eCB-like" lipogenic effects of CBG and CBGV, and examined the effect of co-administration with the eCB AEA. Interestingly, we found that CBG and CBGV did not increase the lipogenic effect of AEA, and in fact, they significantly reduced it in 20 (CBGV) and 10-20 μM (CBG). This suggests that CBG and CBGV may be weak partial activators of the AEA lipogenic pathway.

CBC, CBDV and THCV reduce AA-induced, acne-modeling, pathologically enhanced lipogenesis

As mentioned above, an important step in the pathogenesis of acne is the increase of sebaceous production, as well as a change in its composition. Normalization of these changes is therefore an important goal in the treatment of the disease. Accordingly, we further investigated the effects of THCV, CBC, and CBDV on AA-induced, "acne-like", pathologically enhanced lipid synthesis. Both CBC and

CBDV were found to reduce AA-induced lipid synthesis, but these effects were far surpassed by THCV, which, like CBD, completely normalized sebaceous lipid production. Given that THCV was found to be the most potent of the three pCBs based on these results, we investigated the potential anti-acne effects of non-cytotoxic concentrations of THCV in our subsequent experiments.

THCV exerts a universal lipostatic effect at non-cytotoxic concentrations

In the following experiments, we determined whether this remarkable lipostatic effect of THCV was limited to the prevention of AA-induced, predominantly protein kinase C δ-mediated lipid production, or by activation of a universal lipostatic pathway. We performed Nile Red labeling using two eCBs, AEA and 2-AG, acting on two lipogenic pathways other than AA. We found that, like CBD, THCV is able to normalize the lipogenic effect of both eCBs, i.e., it has a universal lipostatic effect.

Non-cytotoxic concentrations of THCV are anti-proliferative in human sebocytes

In addition to the increased lipid production of individual sebocytes, the proliferation of cells plays a key role in the development of acne-related seborrhea (due to the holocrine secretory mechanism). As such, reducing proliferation alone can be highly effective in reducing sebum production, and as a next step in our experiments, we investigated the effects of THCV on cell numbers. Using the CyQUANT proliferation assay, we demonstrated that THCV reduced the proliferation of sebocytes in a dose-dependent manner over a concentration range of 1-10 μM.

THCV has a potent anti-inflammatory effect

In addition to pathologically increased lipogenesis and proliferation, the third important sebocyte-specific step in acne pathogenesis is inflammation, and therefore,

the effects of THCV on the immune profile of sebocytes were investigated. The inflammatory response was induced using the TLR4 activator lipopolysaccharide (LPS; 5 μg/ml), and measured by the detection of certain "acne-relevant" inflammatory cytokines (namely interleukin [IL]-1α, IL-1β, IL-6, IL-8 and tumor necrosis factor [TNF]-α). THCV was capable of suppressing the increase of these cytokines upon LPS treatment on the mRNA (Q-PCR; IL-1α, IL-1β, IL-6, IL-8, TNF-α) and for some the protein (ELISA; IL-6, IL-8) level. Therefore, THCV (at least *in vitro*) positively influences all sebocyte-specific aspects of acne pathogenesis.

Based on our preliminary results, all pCBs tested have anti-inflammatory activity on human sebocytes

In conclusion of our experiments exploring the effects of pCBs, we investigated the effects of CBC, CBDV, CBG, and CBGV on the immunological behavior of sebocytes. Using the inflammation model already presented for THCV, it was found that all pCBs significantly reduced the effect of LPS on mRNA expression of the above "acne-relevant" cytokines.

NA does not reduce viability up to 100 μM

In the second half of our experiments, we investigated the biological effects of NA, which has been used in clinical practice for decades as a lipid-lowering agent on human sebocytes. Using the MTT assay, we found that NA did not decrease the viability of sebocytes when used in concentrations up to 100 μM even after 24, 48 and 72 hours of treatment.

In order to exclude the possibility of early cell death processes, the effects of NA were further investigated with combined fluorescent $DilC_1(5)$-SYTOX Green. We found that, in accordance with our data obtained with the MTT assay, NA did

not increase the proportion of apoptotic or necrotic cells, confirming that in this time (24-48 h) and concentration (1-100 μM) range it can be used without risk of cytotoxicity.

NA does not affect homeostatic sebaceous lipid production, but normalizes acne-mimicking lipogenesis induced by various lipogenic agents

Subsequently, we examined the effects of NA on the most characteristic biological function of sebocytes, i.e., basal, homeostatic sebaceous lipid production, using fluorescent Nile Red labeling. We found that the use of NA at the above non-cytotoxic concentration does not affect the production of sebum lipids even after 48 hours of treatment.

Given that, as discussed above, one of the key steps in the pathogenesis of acne is abnormally increased sebum production, we next examined whether NA was able to suppress AA-induced acne-mimicking lipid synthesis. We found that the previously identified non-cytotoxic concentrations of NA significantly reduced AA-induced, "acne-like", pathologically increased lipid synthesis.

NA has a universal lipostatic effect

As in the previous series of experiments with pCBs, we were curious to note whether this remarkable lipostatic effect of NA is limited to the normalization of AA-induced, predominantly PKCδ-mediated lipogenesis, or through activation of a universal lipostatic pathway. To address this issue, lipogenic agents (a combination of AEA already mentioned and linoleic acid [LA] and testosterone [T]) were used which, according to literature, enhance other signaling pathways (AEA: CB_2; LA+T: PPARs). We found that both AEA and LA+T significantly increased sebaceous lipid production, whereas NA was able to counteract this effect in both cases. Based on these results NA is likely to have a universal lipostatic action.

NA reduces the proliferation of human sebocytes

Based on the effects described above, it can be concluded that NA may be effective in the treatment of disorders associated with sebaceous gland hyperactivity (such as seborrhea or acne). Because sebum is produced via holocrine secretion, the actual amount of sebum depends not only on the lipid production, but also on the number of individual cells, the efficacy of an anti-seborrheic drug is greatly increased, if not only has lipostatic, but also anti-proliferative properties. Following this logic, the effects of NA on cell numbers were examined using the CyQUANT proliferation assay, and showed a small but significant reduction in sebocyte proliferation over the concentration range of 10-100 μM. It is also noteworthy that, in accordance with the viability data, the cell number did not fall below the 24-hour control level, confirming that NA was indeed not cytotoxic, but "purely" anti-proliferative.

NA does not protect the LPS-induced inflammatory response of sebocytes

As discussed earlier, inflammation is another important factor in the development of acne besides pathologically increased sebaceous lipid production. With this in mind, we investigated how NA influences the immune profile of sebocytes as a conclusion of our phenomenological experiments.

An immunogenic response was induced as above, with 5 μg/ml LPS, followed by cytokine expression and release experiments (IL-1α, IL-1β, IL-6, IL-8 and TNF-α; Q-PCR; and IL-6 and IL-8; ELISA; 3- and 24-hour treatments).

We found that NA at 100 μM did not reduce the LPS-induced increased production of these cytokines, nor did it have a significant effect on gene expression when used alone. We also examined how NA influences the release of two key cytokines, IL-6 and IL-8, through which recent data suggest that sebocytes are

involved in the regulation of Th_{17} cell activity. Consistent with mRNA data, we did not show any NA-dependent effect on cytokine release in either short- (3-hr) or long-term (24-hr) treatments, suggesting that NA does not directly influence the inflammatory and immunological processes of human sebocytes.

The lipostatic effect of NA occurs in a Ca^{2+}-dependent manner

We next decided to investigate the possible mechanisms underlying the above observed effects. Since elevation of $[Ca^{2+}]_{IC}$ often leads to universal lipostatic effects very similar to those described above, and that NA has been shown to increase intracellular calcium in certain cell types, the question arose whether increase in $[Ca^{2+}]_{IC}$ plays a role in mediating the lipostatic effects of NA. To answer this question, we first repeated the lipid synthesis assay in the presence of BAPTA AM a Ca^{2+} chelator. We found that the lipostatic effect of NA was not evident in the presence of 10 μM BAPTA AM, suggesting that Ca^{2+}-linked signaling pathways may play a role in this effect.

NA produces Ca^{2+} signal in human sebocytes, which is predominantly from the extracellular space, but is not TRP channel-mediated

Since, based on the results described above, it seemed likely that NA could induce Ca^{2+} signaling in sebocytes, the next step was Fluo-4 AM labeling. Effective lipostatic concentrations of NA were found to increase $[Ca^{2+}]_{IC}$ in a concentration-dependent manner.

Our next question was to identify the origin of the Ca^{2+} signal observed in the previous experiment. Performing the experiment in a nominally Ca^{2+}-free medium, we found that the reduction of $[Ca^{2+}]_{EC}$ almost completely prevented the Ca^{2+} signal, suggesting that NA activates cell surface Ca^{2+} channels directly or indirectly on human sebocytes. NA is known to activate certain transient receptor potential (TRP)

channels of the vanilloid subfamily, which are mainly Ca^{2+}-permeable, and that several of these channels (TRPV1, TRPV3, and TRPV4) are effective in reducing sebaceous lipid production.

In order to investigate the possible involvement of these channels in the process, our experiments were also performed in the presence of the general TRP channel antagonist ruthenium red (RR). We found that RR did not influence NA-induced calcium signaling, suggesting that unidentified non-TRPV cell surface Ca^{2+} channels may be responsible.

Sebocytes express the primary receptor of NA, HCA_2

In the light of the data presented above, the involvement of TRPV channels in mediating the lipostatic effects of NA seemed unlikely, so we next investigated the possible role of HCA_2, considered as the primary receptor of NA. Q-PCR and immunofluorescent labeling revealed that HCA_2 is present at both mRNA and protein levels in human sebocytes.

Selective gene silencing of HCA_2 prevents NA's lipostatic and $[Ca^{2+}]_{IC}$ increasing effects

Given that no effective and selective antagonists are available against the HCA_2 receptor, siRNA transfection-mediated selective silencing was used, resulting in a significant decrease in HCA_2 expression at both mRNA and protein levels. After verification of the success of gene silencing in SCR-transfected and HCA_2 gene-silenced sebocytes, we found that the cellular effects of NA were indeed HCA_2-mediated, as the lipostatic effect of NA and the Ca^{2+} signal it induced were also reduced.

Neither NA nor AA affect HCA_2 receptor expression

Based on these results, HCA_2 is a novel, previously unknown regulator of human sebaceous lipid production, suggesting that changes in its expression and activity may play a role in mediating the effects of lipogenic agents such as AA. To conclude, we investigated how AA and NA treatments affect HCA_2 expression in human sebocytes. We found that none of the treatments had a significant effect on protein expression of the receptor.

Discussion

Acne is one of the most common human skin diseases, which, although not a life-threatening condition, can significantly impair patients' quality of life. Given that the use of the most effective treatments currently available (such as oral isotretinoin) may lead to the development of serious adverse reactions, it is understandable that significant worldwide efforts are being made to develop new anti-acne drugs that are both safe and effective.

An overview of the effects of selected non-psychotropic pCBs

The ECS has a complex effect on the biological processes of human sebocytes: treatment with eCBs and enhancement of the eCB tone both increase sebaceous lipid production, whereas activation of the ionotropic cannabinoid receptors TRPV1, TRPV3, and TRPV4 reduces it. We have also shown that the use of CBD, the most studied and prescribed pCB, leads to the development of complex cellular anti-acne effects which, in the light of the available clinical trial data, are highly likely to occur *in vivo* as well. In light of these facts, we investigated in the first half of the present study whether non-psychotropic pCBs may have anti-acne effects similar to that of CBD. First, we determined that all five pCBs tested showed apoptosis-dominated cell death at 50-100 μM, but at ≤10 μM, they could be used without the risk of cytotoxicity. It was also shown that pCBs at these non-cytotoxic concentrations differentially influenced lipid synthesis. CBG and CBGV exhibited a small, but significant increase in lipogenesis in an "eCB-like" manner; "CBD-like" CBDV had no significant effect, whereas lipostatic CBC and THCV reduced basal sebaceous lipid synthesis. These results suggest that CBG and CBGV may have a beneficial effect on dry skin disorders, whereas CBC and especially THCV may have anti-acne effects. We found that CBC, CBDV and THCV, both at non-cytotoxic concentrations, significantly reduced the lipogenic effect of AA. Further examination

of the effects of THCV, which is the most efficient of the three pCBs, showed that it is likely that it activates a universal lipostatic signal pathway similarly to CBD, since it normalized not only AA-induced, but also eCB-dependent lipid production. Given that non-cytotoxic, effective lipostatic concentrations of THCV produced a dose-dependent anti-proliferative effect, our data suggested that THCV could effectively reduce sebum production, even *in vivo*. In the next phase of our experiments, we demonstrated that THCV is highly effective in reducing the LPS-induced inflammatory response of sebocytes not only at mRNA, but also at protein level. Overall, therefore, our results showed that THCV positively influenced all sebocyte-specific aspects of acne with comparable efficacy to CBD.

As discussed above, our research has revealed interesting functional heterogeneity among pCBs. In contrast to the other three substances, CBG and CBGV behaved in an "eCB-like" manner, and slightly, but significantly increased sebaceous lipid synthesis in sebocytes. Given that our data obtained at mRNA level indicate that CBG and CBGV (as well as CBC and CBDV) are effective in reducing the LPS-induced inflammatory response of sebocytes, our results suggest that these compounds may be useful in the treatment of certain dermatites and inflammatory conditions.

In terms of clinical translability of our results, it is worth noting that pCBs are well-tolerated even when administered orally, and topical CBD has been shown to be extremely safe in human studies. Given that all pCBs are highly lipophilic compounds, they are likely to enter the skin via the transfollicular route following topical application, and are likely to accumulate in lipid-rich SGs. These physicochemical properties make pCBs ideal candidate drugs for selectively targeting SGs, which can minimize the risk of systemic side effects.

Overview of the effects of NA on human sebocytes

In the second half of our experiments, we focused on the biological effects of NA. We showed that NA at concentrations up to 100 μM did not affect the viability of sebocytes. NA did not induce early cell death processes, and did not affect basal, homeostatic sebaceous lipid production. At the same time, it normalized AA-induced enhanced lipid synthesis. In addition, the lipostatic effect of non-cytotoxic concentrations of NA was universal, as it prevented the action of not only AA, but also AEA (CB_2) and LA+T combinations (PPARs) that activate partially independent signaling pathways. Finally, it has also been shown that the above non-cytotoxic concentrations of NA reduced the proliferation of sebocytes in a concentration-dependent manner, suggesting that it may be effective in the normalization of pathologically increased sebum production *in vivo*.

To investigate the mechanism of the observed beneficial effects, we first investigated the effects of NA on Ca^{2+} homeostasis since, according to our work and others, elevated $[Ca^{2+}]_{IC}$ may lead to lipostatic effects similar to those seen now. We found that the effect of NA on lipid synthesis was indeed Ca^{2+}-dependent, since the effects described above were abrogated in the presence of BAPTA-AM, a Ca^{2+}-chelator. We also demonstrated that lipostatic concentrations of NA increase the $[Ca^{2+}]_{IC}$ of human sebocytes in a concentration-dependent manner, whereas the source of the Ca^{2+} signal is the extracellular space, since the Ca^{2+} signal was almost completely eliminated when nominally Ca^{2+}-free solution was used. It is known that NA is capable of directly activating a number of predominantly Ca^{2+}-permeable TRPV channels, which may exert a lipostatic effect similar to that seen in case of NA administration, therefore, we investigated whether these channels may contribute to the effects of NA. Given that NA-induced Ca^{2+} signal was not reduced by RR, a

general antagonist of TRPV channels, our results suggested that this effect may be due to a previously unknown, TRPV channel-independent mechanism.

Further investigation of the possible mechanism has established the primary receptor of NA, HCA_2, which we showed to be expressed at both mRNA (Q-PCR) and protein (IF) levels in sebocytes. In addition, selective "gene silencing" of the receptor by siRNA transfection prevented both lipostatic effects and Ca^{2+} signaling. Thus, our present data not only raise the potential for topical application of NA, which has been safely used in the clinic for decades, in acne and seborrhea, but also highlight the role of a previously unknown regulator, the HCA_2 receptor, which may be an entirely new therapeutic target for these diseases.

Of course, the therapeutic potential of our results should be explored in well-designed, placebo-controlled, double-blind clinical trials. Although our team is not aware of such studies, in addition to the data presented above, there is ample evidence to support the notion that NA may be beneficial in acne and seborrhea. This evidence includes, firstly, the fact that pellagra (i.e., NA deficiency disease) is frequently characterized by "dyssebacia", i.e., hyperplasia of sebocytes, altered sebum production, and consequent increase in SGs. Secondly, side effects of NA treatment at the pharmacological dose include dry skin. Third, the results of some (non-placebo controlled) pilot clinical trials suggest that oral NA can effectively reduce acne.

It is also worth noting that another member of the vitamin B3 complex, nicotinamide, has been shown to effectively reduce sebum production in a double-blind, placebo-controlled clinical trial, and overall there is growing evidence that it may be effective in treating acne and other skin conditions. Although the mechanism of action of nicotinamide against acne is only partially understood, it is known that it does not activate the HCA_2 receptor, and thus does not activate the lipostatic signaling pathway described above in connection with NA. However, given that NA

can be converted to nicotinamide *in vivo*, the clinical efficacy of topical NA may even exceed that of nicotinamide, since it can theoretically produce both HCA_2-dependent direct and HCA_2-independent, indirect anti-acne effects.

Interestingly, despite its lipostatic and anti-proliferative efficacy, NA was unable to prevent the LPS-induced inflammatory response of sebocytes. It is worth noting, however, that based on our previous data obtained with CBD testing, the lack of anti-inflammatory effect of NA is not completely unexpected. According to our previous results, an independent anti-inflammatory pathway (adenosine A_{2A} receptor → cAMP↑ → TRIB3↑ → inhibition of p65 NF-κB) is responsible for the relevant effects of CBD, while the lipostatic and anti-proliferative effects depend on the activation of TRPV4 ion channels and consequent Ca^{2+} signaling. In addition, it has recently been shown that activation of another Ca^{2+}-permeable ion channel, namely TRPV3 (besides, just like TRPV1 and TRPV4, decreasing acne-mimicking excessive sebaceous lipid production) increased expression and release of several pro-inflammatory cytokines both in human sebocytes and keratinocytes. With these in mind, it is not surprising that NA, through activation of HCA_2 and subsequent elevation of $[Ca^{2+}]_{IC}$, effectively normalized sebaceous lipid synthesis, but was unable to counteract the inflammatory response to LPS.

Although our results suggest that HCA_2 is a novel regulator of SG biology, an important question remains at the end of our experiments, namely how HCA_2 receptor activation can lead to $[Ca^{2+}]_{IC}$ increase in human sebocytes. Although HCA_2 is a G_i-coupled receptor, it is not unprecedented in the literature for its activation to be followed by Ca^{2+} influx. Identification of the currently unknown Ca^{2+}-permeable pore would be important because its direct pharmacological activation would add a new therapeutic target to treat acne and seborrhea. It should also be noted that, in contrast to TRPV3, where activation of the ion channel leads to the formation of an

inflammatory response in addition to the lipostatic effect, this unknown Ca^{2+} channel has been shown to influence the Ca^{2+} homeostasis of sebocytes in a manner that does not elicit an inflammatory response.

Concluding thoughts - Limitations of this study

Although promising experimental data has been presented above, the translational value of our study is limited by the fact that our findings are based on a single cell line due to the unfortunate lack of appropriate animal models and the objective difficulty of maintaining primary sebocyte cultures. However, since the cell model we have used to date has reliably predicted clinical efficacy in CBD, further studies of both pCB and NA in well-designed, randomized, double-blind, placebo-controlled clinical trials are warranted. These compounds hold promise to develop new drugs with a fundamentally novel mechanisms of action.

Summary

In the first half of our experiments, we aimed to investigate the biological effects of certain non-psychotropic pCBs on human SZ95 sebocytes. We have shown that pCBs up to 10 μM have different effects on the most typical biological function of sebocytes, i.e. basal sebaceous lipid synthesis, without reducing cell viability. CBG and CBGV increased lipogenesis similarly to eCBs, CBDV had no significant effect, whereas CBC and THCV decreased basal sebaceous lipid synthesis. Because all pCBs were effective in reducing the LPS-induced inflammatory response of sebocytes, these results suggest that CBG and CBGV may have a beneficial effect on dermatitis-associated inflammatory disease.

Continuing our experiments, we found that CBC, CBDV and THCV were able to significantly reduce AA-induced lipogenesis. Further examination of THCV, which was found to be the most effective of the above, found that it behaves as a "universal" lipostatic agent (i.e., it is also capable of blocking the action of lipogens acting on signaling pathways other than AA), is anti-proliferative, and almost completely normalizes LPS-induced expression of inflammatory cytokines.

In the second half of our experiments, NA was found to effectively reduce sebocyte proliferation without affecting viability and homeostatic sebaceous lipid production, as well as pathologically enhanced sebaceous lipid synthesis induced by various lipogenic agents. It has also been shown that the effects of NA are mediated by HCA_2 receptor activation and consequent Ca^{2+} influx.

Taken together, our results suggest that both NA and pCB have complex effects on the biological processes of human sebocytes, and may be promising new tools for treating various FM dysfunctions (dry skin, acne, seborrhea, etc.)

Acknowledgement

Hereby I would like to thank all those who contributed in any way to the dissertation. First of all, I would like to thank Prof. Dr. László Csernoch, head of the Department of Physiology of the University of Debrecen, who provided the necessary infrastructural background for my work. I thank my supervisor Prof. Dr. Tamás Bíró and Dr. Attila Oláh for their professional and human support over the years, for their trust in me, and for giving me both important and professional examples and learning a great deal from them.

I would like to thank the former and current staff of the lab and the institute, PhD and undergraduate students, who were always there to help me when needed. Even among them, my greatest gratitude goes to my closest colleagues, namely to Dr. Johanna Mihály, Dr. Balázs Tóth, Dr. Gábor Szöllősi Attila, Dr. Gabriella Czifra, Dorottya Ádám, Dr. Alimohammadi Shahrzad, Dr. Lídia Ambrus, Ágnes Angyal, Norbert Balogh, Dr. Erika Lisztes, Kinga Fanni Tóth, Dr. Imre Lőrinc Szabó, Dr. Nikolett Vasas, Anita Vladár, Dr. Nóra Zákány, Szilvia Bánhalminé Szilágyi, Nikolett Fricz, Lilla Furin, Anita Galicz, Erika Hollósi, Judit Szabó-Papp, Renáta Uzonyi, Gergő Kovács, Marietta Margit Budai, Dr. Erika Takács, Katalin Eszter Sós, and Dr. Pálma Tímea Szabó.

I thank the work of my undergraduate student Dr. József Magi, who has proven himself to be a very talented, hard-working student committed to science. It always makes me happy remembering the time we spent together in the work.

Thank you for the invaluable help of our collaboration partners (Dr. Colin Stott, Prof. Dr. Christos C. Zouboulis, Prof. Dr. Zoltán Benyó, Dr. Adrienn Gyöngyösi, Tímea Szendi-Szatmári and many others).

Finally, I thank all my parents and friends. I am very grateful to them, because without them I would not have made it.

The research was supported by the National Talent Program of the Ministry of Human Resources (NTP-NFTÖ-16-0463), as well as by GINOP-2.3.2-15-15-2016-00050, and it was realized within the confines of the "Lendület" program of the Hungarian Academy of Sciences (LP2011-003/2015).

Appendix – List of publications

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Subject: PhD Publikációs Lista

Candidate: Arnold Markovics
Neptun ID: GGHZGV
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Markovics, A.**, Tóth, K. F., Sós, K., Magi, J., Gyöngyösi, A., Benyó, Z., Zouboulis, C. C., Bíró, T., Oláh, A.: Nicotinic acid suppresses sebaceous lipogenesis of human sebocytes via activating hydroxycarboxylic acid receptor 2 (HCA2).
J. Cell. Mol. Med. 23 (9), 6203-6214, 2019.
DOI: http://dx.doi.org/10.1111/jcmm.14505
IF: 4.658 (2018)

2. Oláh, A., **Markovics, A.**, Papp, J., Szabó, P., Stott, C., Zouboulis, C. C., Bíró, T.: Differential effectiveness of selected non-psychotropic phytocannabinoids on human sebocyte functions implicates their introduction in dry/seborrhoeic skin and acne treatment.
Exp. Dermatol. 25 (9), 701-707, 2016.
DOI: http://dx.doi.org/10.1111/exd.13042
IF: 2.532

List of other publications

3. Biró, A., **Markovics, A.**, Homoki, J., Szőllősi, E., Hegedűs, C., Tarapcsák, S., Lukács, J., Stündl, L., Gálné Remenyik, J.: Anthocyanin-Rich Sour Cherry Extract Attenuates the Lipopolysaccharide-Induced Endothelial Inflammatory Response.
Molecules. 24 (19), 3427-3441, 2019.
DOI: http://dx.doi.org/10.3390/molecules24193427
IF: 3.06 (2018)

4. Zákány, N., Oláh, A., **Markovics, A.**, Takács, E., Aranyász, A., Nicolussi, S., Piscitelli, F., Allarà, M., Pór, Á., Kovács, I., Zouboulis, C. C., Gertsch, J., Di Marzo, V., Bíró, T., Szabó, T.: Endocannabinoid tone regulates human sebocyte biology.
J. Invest. Dermatol. 138 (8), 1699-1706, 2018.
DOI: http://dx.doi.org/10.1016/j.jid.2018.02.022
IF: 6.29

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5. Paholcsek, M., Leiter, É., **Markovics, A.**, Biró, S.: Novel and sensitive qPCR assays for the detection and identification of aspergillosis causing species.
Acta Microbiol. Immunol. Hung. 61 (3), 273-284, 2014.
DOI: http://dx.doi.org/10.1556/AMicr.61.2014.3.3
IF: 0.778

Total IF of journals (all publications): 17,318
Total IF of journals (publications related to the dissertation): 7,19

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

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