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

Investigation of the Physiological Function and Pharmacological Manipulation of Transient Receptor Potential Vanilloid Ion Channels on Human Hair Follicles

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The Examination takes place at the Department of Biophysics and Cell Biology, Faculty of Medicine, University of Debrecen
November 18, 2022, 11:00

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November 18, 2022, 13:00

Introduction and literature review

The integumentary system of the human body contains numerous appendages, of which the hair follicle (HF) plays an important role in the skin's barrier integrity as well as in its hormonal, psychosocial, and sensory functions. The unique and repetitive life cycle of the HF is termed the “hair cycle”, which consists of recurring proliferative (hair-forming), regressive, and quiescent phases. The regulation of the hair cycle is a complex process in which many systemic hormones and paracrine mediators participate. HFs form structural and functional unit with sebaceous glands within the skin (pilosebaceous unit, PSU) that is of further (patho)physiological significance.

Our research team investigates the relationship between the skin and the endocannabinoid system (ECS) and the closely related transient receptor potential (TRP) ion channel superfamily members. In addition, we also examine the effects of pharmacological manipulation of the ECS by *Cannabis Sativa* plant derived compounds, the so-called phytocannabinoids in the skin. According to our earlier results, the ECS plays important roles in the regulation of the hair cycle, i.e., the activation of both TRP Vanilloid 1 (TRPV1) and TRPV3 ion channels and the metabotropic CB1 cannabinoid receptor induced (regressive) catagen phase. Based on these findings, it is assumed that a pro-catagen ECS-tone is established on the hair cycle. Hereby, we introduce another TRP ion channel, TRPV4 as a novel hair cycle regulator in human, as assessed by *in vitro* and *ex vivo* experiments.

Also, based on earlier results of our team, it is known that a non-psychoactive phytocannabinoid, cannabidiol (CBD) exerts complex anti-acne effects, so it may also be a promising agent in acne therapy. Given the HF’s close structural and functional relationship to sebaceous glands, HFs also contribute to the pathogenesis of acne. Thereby, it is important to extend analyzing the effects of CBD to HFs as well. Therefore, we studied the effects of CBD on the hair cycle and in an *in vitro* model of inflammation. In addition, we investigated whether

CBD activates potential receptors associated with it (TRPV4 and adenosine receptors) and that are already known to be expressed on HFs.

The structure and change of HF morphology during the life cycle of the HF, the "hair cycle"

The HF resembles an onion, both morphologically and structurally (due to the concentric epithelial layers around the central hair shaft). This morphology, however, changes to a certain degree during the special life cycle of HF, the hair cycle. Among the three phases of hair cycles, the "anagen" phase is the active stage of formation and growth of hair. Then, the hair follicle undergoes apoptosis driven regression, this "catagen" phase is followed by a resting "telogen" phase. The hair cycle is repetitive, meaning that a growing active HF will ultimately undergo regression, but the same gives the possibility that after the loss of a hair shaft, a new hair can develop by re-entering a new anagen phase. During the different phases of the hair cycle, HF morphology and cell processes in different compartments change in a uniform fashion. Some parts of HF, e.g., the otherwise intensively proliferating and hair shaft producing matrix keratinocytes (MK) undergo apoptosis during catagen transition. In contrast, the vascularized and innervated central regulatory region surrounded by MKs in the middle of the bulb, the dermal papilla (DP), remain intact during the changes of hair cycles.

Hair cycle regulation

Numerous hormones are involved in the regulation of the hair cycle, however, human HFs do not switch synchronously between the phases of the hair cycle. Therefore, paracrine mediators between different compartments of the HF play crucial roles in the regulation of the hair cycle. Several paracrine mediators, mainly cytokines and growth factors (e.g., pro-catagen TGF-β and EGF, or IGF that is important in anagen maintenance) have been described to date in the regulation of the hair cycle.

Adenosine differs somewhat from the aforementioned paracrine factors, i.e., it is not a secreted polypeptide but a purine nucleotide formed during metabolic processes. In addition to its metabolic role, it is also ligand of its dedicated membrane receptors and exerts ubiquitous anti-inflammatory effects. HFs express adenosine receptors ($A_{1,2a-2b,3}$), and adenosine treatment enhance hair growth via A_{2b} activation which is partly mediated through the DP cells. Previous results of our research group revealed that ORSKs contribute to the effects of adenosine by manipulating the level of hair cycle regulatory factors (eg. TGF-β2 shifting, IGF1R, EGF) and anagen propagation. The effect of minoxidil, a hair growth enhancer used successfully in the clinical practice, is known to increase the release of adenosine.

Immunological aspects of HF biology, inflammation of the HF

The immunological significance of the skin originates partly from its barrier function; however, it contains a significant number of resident immune cells, moreover, even keratinocytes are also able to perform effector immune functions under certain conditions. Components of the innate immune system are also present in the HF. Pathogenic microbes exhibit typical molecular patterns generally not present on eukaryotes (e.g., viral double-stranded RNA) that can be recognized by receptors known as "Toll-like receptors" (TLRs) that are present in abundance in cells of the skin. Upon activation, they lead to increased expression of antimicrobial proteins as well as pro-inflammatory cytokines, e.g. of interleukin-6 (IL-6). The locally released cytokines exert not only proinflammatory effects, but they fundamentally influence HF biology. IL-1α, IL-1β, and TNFα can inhibit the growth of human HFs *in vitro,* resulting in a dystrophic anagen phase.

The transient receptor potential (TRP) ion channel superfamily and the endocannabinoid system (ECS) – their impact on human skin physiology

The transient receptor potential (TRP) ion channel superfamily is a group of evolutionary conserved cation channels. The 28 known mammalian TRP channels are divided into 8 sub-families: the vanilloid (TRPV), melastatin (TRPM), canonical (TRPC), policystin (TRPP), mucolypin (TRPML) and ankyrin (TRPA). Interestingly, certain TRP channels are sensitive to heat stimuli, in addition, these "thermo-TRP"-channels appear to be especially important in the regulation of physiological functions of the skin. Of these TRP channels, TRPA1 and TRPM7 can be activated by cold, TRPV3 and TRPV4 by warm, whereas TRPV1 and TRPV2 by hot thermal stimulus.

TRPV4 is a non-selective Ca^{2+}-channel formed by four subunits, each containing 6 TM domains. It usually occurs in homotetrameric form, but combinations formed with other TRP channel subunits (e.g., TRPC1, TRPP2) have also been described. The TRPV4 channel can be activated by various modalities, including osmolarity change and mechanical deformation. TRPV4 is a thermo-TRP channel and it can be activated by temperatures above 25 ^{o}C, but the threshold temperature may vary. Thermal sensitivity can be affected by various conditions (activated signaling pathways, sensitization with other stimuli, heteromerization with other thermo-TRP channels), ultimately, the threshold temperature might be close to skin surface temperature. The complex temperature dependence of the channel is characterized by an inactive state due to desensitization and accommodation. Thus, the activation of TRPV4 by thermal stimuli depends on several factors, and although this has not been studied *in vitro* in isolated human keratinocytes, the TRPV4 activation threshold temperature in mouse keratinocytes was measured to be approximately 32 ^{o}C.

TRPV4 function is increasingly associated with the development of inflammation, because in case of its pharmacological or genetic inhibition, LPS-induced inflammatory cytokine production is lacking or significantly reduced. Low pH and various acidic compounds can also activate TRPV4, which can accompany

inflammatory processes. Among the endogenous ligands, the endocannabinoid anandamide, and its metabolite, arachinoid acid are both able to activate TRPV4. TRPV4 may play an important role in the maintenance of the epidermal barrier, as a participant of cell-cell connections in the zonula adherens. It also contributes to the formation of zonula occludens-type connections, which also enhances barrier functions by limiting paracellular fluid and ion loss through the skin. Raising the temperature stimulates recovery of the damaged barrier *in vivo* in both mice and humans, an effect that is thought to be mediated by TRPV4 activation. TRPV4 has a role in vasodilation of skin vessels, so it may also play a role in thermoregulation. Activation of TRPV4 expressed in sebaceous gland cells inhibits their proliferation. TRPV4 expression and the effect of its activation in the hair follicle has not been studied yet, but the results of experiments on mouse skin showed TRPV4 expressed predominantly in the ORS layer and its expression could not be confirmed in the DP.

The endocannabinoid system and its role in the physiological function of the skin

The endocannanbinoid system (ECS) is a complex signaling network that includes dedicated receptors, their endogenous ligands, the so-called endocannabinoids (eCBs), and the enzyme apparatus required for their synthesis and degradation. Two receptors that are classically linked to ECS are cannabinoid receptor-1 and -2 (CB1 and CB2), but other "novel receptors" have also been described. In a broader sense, ECS also includes receptors or enzymes that are otherwise better associated other signaling systems but also mediate eCB effects.

The ECS plays important roles in the skin, including the maintenance of the epidermal barrier, melanogenesis, immune responses, and is involved in the perception of pain and itch in peripheral nerves. In the skin appendages, CB2 is expressed on the sebaceous gland, and it mediates lipid synthesis-enhancing effects. The hair follicle is of further importance because it is also capable of synthesizing endocannabinoid molecules (mainly anandamide). Activation of the CB1 receptor expressed on HFs leads to catagen induction *ex vivo* (whereas the

CB2 receptor is not expressed on HF in human). In addition, the prototypical TRP receptor, TRPV1, is also expressed on HFs.

Plant derived cannabinoid compounds (phytocannabinoids) and their possible use in dermatology

The *Cannabis sativa* plant produces numerous so-called phytocannabinoids (PKs), of which the best characterised is (-)-trans -Δ^9-tetrahydrocannabinol (THC) that is responsible for its psychotropic effects. To the best of our knowledge, out of thousands of different molecules that can be identified in a *C. sativa*, approx. 120-150 are PKs, i.e., substances that are able to exert effects through ECS manipulation. While the use of THC is limited by its above-mentioned psychoactive nature, several other PKs appear to be promising for clinical use. Of these, research focuses on (-)-cannabidiol (cannabidiol, CBD), which is free of the psychotropic effects associated with THC. Currently, due to conflicting results, there is no consensus on the relationship of CBD to CB receptor. However, CBD's effects are increasingly associated with other receptors explaining its diverse pharmacological effects. CBD has been successfully used systemically in neuropsychiatric disorders, but further intensive research is underway to broaden its clinical use.

CBD treatment enhances the expression of keratins involved in wound healing in rodent skin *in vivo*, and CBD exerts cytoprotective effects on fibroblasts and it was also capable of reversing the effect of UV/B radiation dermis by exerting antioxidant and anti-inflammatory effects. Case reports have been already published about successful application of topical CBD in skin diseases. Previous results from our group shows that CBD reduces abnormal sebum production in an *in vitro* immortalized human sebaceous gland cell line by the activation of TRPV4 and A_{2A} receptors and it is also associated by anti-inflammatory effects. These results suggest that CBD may be effective in conditions associated with abnormal sebum production, e.g., acne.

Objectives

The ECS has already been reported to be highly represented in the PSU, but we lack any information about TRPV4's involvement in regulating biological processes of HFs. Moreover, CBD seems to be a promising molecule in dermatological application. As many targets other than TRPV4 are already described in HFs, we aimed at investigating its effect on human HFs.

Therefore, we aimed at detecting TRPV4 expression and its distribution in human HFs. We also tested the involvement of TRPV4 in hair cycle regulation. We studied the effect of TRPV4 activation on cellular level (apoptosis, necrosis, viability) in HF cell types that abundantly express it, namely, outer root sheath keratinocytes. We also aimed at studying the dynamics of TRPV4-driven calcium influx and its heat-dependence.

We conducted experiments in order to characterize the effects exerted by the CBD application on the hair cycle and the above mentioned cellular processes as well. We aimed at clarifying which receptors mediate the effect of CBD on HFs. We tested whether CBD exerts its well-known anti-inflammatory effect on the human HFs ex vivo.

Materials and methods

Culture conditions and specific agents used during experiments

During cell and organ cultures described below, we used specific and synthetic molecules, GSK1016790A (GSK) to activate and HC067047 to inhibit TRPV4, respectively. The synthetic analogue of natural double-stranded (viral) RNA, polyinosinic:polycytidylic acid [poly (I:C)], was used to activate the TLR3 receptor, while CGS-15943, known as a "pan antagonist" of the adenosine receptor, was used to inhibit adenosine receptors. The solvent for GSK1016790A, HC067047, CGS15943 was dimethyl sulfoxide (DMSO), CBD was dissolved in ethanol and poly (I:C) was dissolved in nuclease free water. Cell and tissue cultures were performed at 37 °C in a humidified atmosphere supplemented with 5% CO_2 partial pressure.

Isolation and maintenance of HF organ cultures

Human skin samples were obtained following written informed consent from healthy individuals undergoing dermato-surgical procedures, adhering to Helsinki guidelines, and after obtaining the Institutional Research Ethics Committee's permission. Human anagen VI HFs (n=18 per group) were maintained in Williams' E medium supplemented with 2 mM L-glutamine, 10 ng/ml hydrocortisone, 10 mg/ml insulin, and antibiotics.

ORS keratinocyte cultures

Anagen HFs epilated from healthy volunteers scalp were digested using trypsin and EDTA to obtain ORS keratinocytes and were kept on a feeder layer of mitomycin-treated HDF, in a 1:3 mixture of Ham's F12 and DMEM supplemented with cholera toxin, insulin, hydrocortisone, adenine, triiodothyronine, epidermal growth factor, ascorbyl-2-phosphate, and antibiotics.

Immunolabeling of TRPV4

Cryostat HF sections or acetone-fixed ORS keratinocytes grown on coverslips were first incubated with primary antibody (1:100, overnight) against TRPV4, followed with Alexa Fluor 488 dye-conjugated secondary antibody according to standard procedures. Nuclei were counterstained with Vectashield mounting medium containing 40-6-diamidino-2-phenylindole (DAPI).

Hair shaft elongation experiments

Isolated HFs were maintained in the above detailed medium that was changed every other day, whereas treatment with TRPV4 activator or CBD was performed daily. Length measurements were performed on individual HFs using a light microscope with an eyepiece measuring graticule along with photodocumentation. Results were expressed as means (+/- SEM) normalized to vehicle-treated controll HFs.

Histology and histomorphometry

Cryostat sections (6 μm) of cultured human anagen VI HFs were fixed in acetone, air-dried, and processed for histology. Hematoxylin and eosin staining was used for studying HF morphology and hair cycle stage (anagen, early and late catagen) of each HF was assessed according to defined morphological criteria.

Ki-67/TUNEL double labeling

To evaluate apoptotic cells in the HFs in colocalization with a proliferation marker Ki-67, a Ki-67/TUNEL double-staining method was used. Cryostat sections were fixed in formalin/ethanol/acetic acid and labeled with a digoxigenin-deoxyUTP (Apoptag kit) in presence of terminal deoxynucleotidyl transferase, followed by incubation with a mouse anti-Ki-67 antiserum according to the manufacturer's protocol. The number of cells positive for Ki-67 and TUNEL IR was counted per hair bulb and was normalized to the number of total (DAPI+) cells.

Quantitative real-time PCR

Quantitative real-time PCR was performed on a Stratagene MXP3005p detection system by using the 5' nuclease assay following total RNA was isolated using TRIzol reagent according to manufacturer's protocol. 3 μg of total RNA was reverse transcribed into cDNA by using 15 U of AMV reverse transcriptase and 0.025 mg/ml random primers. PCR amplification was performed using TaqMan primers and probes with TaqMan universal PCR master mix protocol. As internal control, transcript of glyceraldehyde 3-phosphate dehydrogenase was determined.

Microfluorimetric measurements of $[Ca^{2+}]_{ic}$

ORS keratinocyte cells were seeded in 96-well black-well/clearbottom plates at a density of 20000 cells/well previously coated with 1% collagen and cultured at 37 ^{o}C for 24 h. The cells were then loaded with the 1 μM Fluo-4 AM at 37 ^{o}C for 30 minutes in Hank's balanced salt solution supplemented with 1% BSA and 2.5 mM probenecid. The cells were washed and finally kept in supplemented Hank's balanced salt solution for 30 minutes at 37 ^{o}C. The plates were then placed into a FlexStation II 384 Fluorescence Imaging Plate Reader (FLIPR) and changes in $[Ca^{2+}]_{ic}$ (reflected by changes in fluorescence; λ_{EX}=494 nm, λ_{EM}=516 nm) induced by various concentrations of the drugs were recorded in each well. Experiments were performed in quadruplets and the averaged data (±SEM) were used in the calculations.

Heat induced Ca^{2+}-influx

To determine the temperature-response of TRPV4 expressed on ORS keratinocytes, we utilized a Fluo-4-based assay using a PCR system. ORS keratinocytes were loaded with 2 μM Fluo-4-AM for 30 min in a 35 mm petri dish, trypsinized, centrifuged, resuspended in Hank's balanced salt solution supplemented as above, and transferred to a 96-well reaction plate (at 100,000 cells/well). Following two minutes of incubation at 25 ^{o}C, Fluo-4 fluorescence was measured with FAM filter set (λ_{EX}=492 nm, λ_{EM}=516 nm) while the well temperature was raised from 25 ^{o}C to 31 ^{o}C and kept at 31 ^{o}C for 25.5 minutes. Background-subtracted fluorescence signals were calculated as a difference of

normalized fluorescence intensity of the ORSKs and Hank's balanced salt solution containing 2 μM Fluo-4 AM. The TRPV4 antagonist HC067047 was applied at 10 μM concentration solution.

Evaluation of cellular proliferation

Cells (5,000 cells/well) were cultured in 96-well black-well/clear-bottom plates in octuplicates and were treated with different concentrations of CBD for the time indicated. Supernatant were then removed by blotting on paper towels, and the plates were subsequently frozen at -70 ˚C. The plates were then thawed at room temperature, and 200 μl of CyQuant GR dye/cell lysis buffer mixture was added to each well. After 5 minutes of incubation, fluorescence was measured at 490 nm excitation and 520 nm emission wavelengths using a Flexstation III plate reader.

Evaluation of cellular viability

ORSKs were seeded in 96-well plates (density: 10,000 cells/well). Octuplicates were treated for 3 hours, after which cells were incubated with 0.5 mg/ml MTT solution for 2 hours, followed by dissolving the freshly formed formazan crystals – the quantity of which correlates to the viable cell number. The absorbance was measured at 567 mm utilizing a Flexstation III plate reader, and the results were expressed as percentage of vehicle control regarded as 100 %.

Determination of necrosis

Cells were seeded (density: 10,000 cells/well) in 96-well black-well/clear-bottom plates, and octuplicates were treated with GSK1017490A (GSK) or CBD for 3 hours. After discarding supernatants, cells were incubated with 1 μM SYTOX green dye for 30 minutes followed by a PBS wash. The fluorescence emission of SYTOX Green dye was monitored by a FlexStation III fluorescent plate reader at 545 nm excitation and 590 nm emission wavelengths. The results were expressed as percentage of vehicle control regarded as 100%.

Determination of apoptosis

Apoptotic process was detected using MitoProbe DilC1(5) Assay Kit. Cells were seeded (density: 10,000 cells/well) in 96-well black-well/clear-bottom plates, and octuplicates were treated with GSK1017490A or CBD for 3 hours. After discarding supernatants, cells were incubated with DilC1(5) solution for 30 minutes and the fluorescence of the dye was measured at 630 nm excitation and 670 nm emission wavelengths using a Flexstation III fluorescent plate reader. The results were expressed as percentage of vehicle control regarded as 100 %.

Statistical analysis

When applicable, data were analyzed using a two-tailed unpaired t-test or ANOVA with Bonferroni post hoc test and P=0.05 values were regarded as significant differences.

Results

1. Expression, distribution, and function of TRPV4 ion channel in HF compartments

The expression and distribuition of TRPV4 in human HFs

TRPV4 was detectable at mRNA levels in both human hair follicles and primary cultures of human ORSKs. Based on immunofluorescence labeling, a significant amount of TRPV4 was present in the epithelial compartments, predominantly in the ORS layer, to a lesser extent in the inner root sheath. There was no obvious TRPV4 expression in the intensely proliferating MK region or in the DP regulating the hair cycle. In the MK region, those layers showed positive TRPV4 immunoreactivity (IR) that are continuous with ORS and can be considered as precursors of the ORSKs. There was scarcely detectable positivity in the connective tissue sheet. Primary ORSK cells also expressed TRPV4 protein during *in vitro* culture.

TRPV4 activation inhibited ex vivo hair growth and induced catagen phase in human HF

Chemical activation of TRPV4 with a synthetic and specific ligand, GSK106790A (GSK), significantly and dose-dependently inhibited *ex vivo* hair growth compared to vehicle-only control medium treated HF-group. At high (100 nM <) concentration GSK treatment, HF length decreased and there were marked differences between treatment groups (n = 18 HF) after only two days of treatment. Given that anagen-phase hair follicles can synthesize hair shaft (and HFs in anagen phase VI were selected for elongation experiments initially), the growth inhibitory effect can also be considered as a sign of shift towards catagen phase. When the antagonist was used simultaneously, growth inhibition could be prevented.

The snap-frozen sections of hair follicles after six days in organ culture

experiments were classified to anagen, early catagen, and late catagen phases, based on standard morphological criteria. Correlated with the dose of TRPV4 activator treatment used, the proportion of HFs shifted toward early/late catagen phase HFs. After 6 days of treatment, there was a spectacular shift in HFs treated with TRPV4 activator, so that there was little to no anagen phase HF in the case of HFs treated with 100 nM GSK and 1 μM GSK, respectively.

As a result of TRPV4 activation, proliferation and apoptotic processes of matrix keratinocytes changed in the directions characterizing catagen HFs

Keratinocytes in the matrix of anagen HFs divide intensively, resulting in hair growth. Ki67 immunoreactivity (red), which indicates intense proliferation in the matrix region of untreated HFs perfectly outlined the MK region itself, with most cells (approximately 60–70%) being Ki67-positive. The proliferation of matrix keratinocytes was gradually decreased with increasing dose of TRPV4 activator treatment. In parallel, TRPV4 agonist treatment significantly increased the number of apoptotic TUNEL-positive (green immunoreactivity) cells that were not or only unfrequently seen otherwise in anagen HFs, and it is also characteristic of catagen hair follicles. TRPV4 antagonist treatment HC067047 was able to prevent the decrease in proliferation.

Investigation of TRPV4 function on Ca^{2+} homeostasis of ORSKs

TRPV4 is a non-specific Ca^{2+} -permeable cation channel, i.e., it enables Ca^{2+} to cross the cell membrane through the pore-forming region when it becomes activated. During our experiments, activation was first tested with a chemical stimulus, by the previously used GSK1016790A. Cultures of primary human ORSKs were assayed by Fluo-4 fluorescent Ca^{2+} measurements. Our results showed that the TRPV4 activator increased the intracellular Ca^{2+} concentration ($[Ca^{2+}]_{ic}$) in a concentration-dependent manner. In representative fluorescence measurements, $[Ca^{2+}]_{ic}$ increased almost immediately after TRPV4 activator treatment. In addition to the functionality of TRPV4, we also tested the efficacy of the TRPV4 antagonist HC067047, the co-application of which prevented Ca^{2+}-

influx. Thus, it can be concluded that the changes in HF following the previously seen TRPV4 agonist treatment were indeed due to TRPV4 activation, and TRPV4 functions as a functional Ca^{2+} channel in human ORSKs.

TRPV4 is involved in mediating Ca^{2+} influx in ORSKs as a response to heat stimulus in vitro

Based on prevoious reports from experiments on rodent keratinocytes that seemed most relevant, the cells were exposed to a 31 ^{o}C heat stimulus after an incubation period at 25 ^{o}C. The fluorescence intensity measured on ORSKs increased after the temperature change, i.e., the cells were able to respond to the thermal stimulus with a gradual increase in $[Ca^{2+}]_{ic}$ *in vitro*. TRPV4 contributed to this effect because Ca^{2+} influx was significantly reduced in the presence of the TRPV4 antagonist.

TRPV4 activation did not affect the viability of ORSKs but induced apoptosis in vitro

The aforementioned effects shown in the MKs on HFs (decreased proliferation and increased apoptosis) are features that accompany or follow catagen induction. However, TRPV4 is expressed and functions on keratinocytes in the ORS layer. Upon TRPV4 activation, our results showed that cell viability did not change (MTT assay), and no difference was observed in the SYTOX assay nuclear staining, which correlates with fragmentation on the membrane indicating necrosis. In contrast, TRPV4 activator reduced mitochondrial membrane potential, which is considered an early sign of apoptosis, and GSK treatment correlated with DilC1(5) fluorescence intensity in a concentration-dependent manner.

2. Effects of CBD on biological processes in HF

In the second part of our experiments, we investigated the effects of CBD on intact human HF in *ex vivo* organ culture. In addition to the effects on hair cycle, since many receptors belonging to the potential target spectrum of CBD (TRPV4,

adenosine receptors) are present on HFs, the possible role of these receptors in mediating CBD's effect was also tested on ORSKs.

CBD applied at high concentrations significantly inhibited hair growth, whereas CBD at low concentrations insignificantly increased hair shaft elongation

When applied at high concentrations (10 μM), CBD significantly and almost completely inhibited hair growth, however, when used at the lowest tested concentration (0.1 μM), it slightly increased elongation (however, this effect was not statistically significant for all donors). Growth inhibition induced by CBD developed soon, by the time of the second measurement. Based on our measurements, 1 μM CBD treatment had no effect at all on hair growth in human HFs *ex vivo*.

CBD at high concentration induced catagen phase ex vivo, along with apoptosis of matrix keratinocytes and inhibited proliferation in the matrix

Based on our studies, CBD at low dose did not show a markedly different effect compared to the control group (vehicle-treated HFs), i.e., approximately the same proportion of the initial all-anagen-phase HFs remained in anagen, while a small portion of them entered early (at 1 μM dose, 1 HF late) catagen phase. In contrast, 10 μM CBD treatment dramatically reduced the number and proportion of HFs in the anagen phase. This result is consistent with that observed during the elongation experiment, as catagen hair follicles do not produce hair. The proportion of late catagen hair follicles were dominant in this treatment group.

CBD treatment reduced proliferation in the matrix in a concentration-dependent manner, which was significant at 1 μM concentration, but was dramatic at 10 μM concentration. In parallel, apoptotic processes infrequently observed in anagen HFs were significantly enhanced by CBD at a dose of 10 μM, albeit at a low concentration it tended to decrease it.

CBD at high concentrations reduced the viability and proliferative potential of ORSKs

The effect on cell number and proliferation can also be analyzed using CyQUANT assay based on the quantification of genomic DNA. Based on our results, CBD after 3 hours of treatment did not have a significant effect on ORSKs. However, after 24 hours, CBD reduced the cell number significantly and in a concentration-dependent manner. These effects increased after 48 and 72 h, respectively, with CBD reaching its maximum effect at 10 μM, while treatment with 5 μM CBD had less but still significant effect on cell number. At low concentrations, below 1 μM, CBD treatment had no effect on cell number.

CBD at high concentrations inhibited the viability of ORSKs in a combined manner, inducing both apoptosis and necrosis

During 3 hours of treatment, a significant decrease in cell number was observed only in the case of CBD treatment applied at a concentration of 50 μM. Considering this, DiLC-SYTOX fluorescence intensity values measured at 50 μM CBD treatment were obtained from significantly fewer cells compared to measurements from other treatment groups, which affects the interpretation of these results. CBD caused necrosis above 10 μM and lead to a decrease in mitochondrial membrane potential characteristic of apoptosis even at a low concentration of 1 μM. At concentrations below this (0.1 μM) concentration range, we could not detect any change in the early apoptotic/necrotic processes examined.

High concentrations of CBD led to predominantly TRPV4-mediated Ca^{2+} *influx in ORSK*

CBD treatment was able to increase the $[Ca^{2+}]_{ic}$ of ORSKs in a concentration-dependent manner, which was found to be significant over 10 μM. When the effect of 50 μM CBD was examined in the presence of a TRPV4 antagonist HC067047, we found that Ca^{2+} influx caused by CBD was significantly reduced.

CBD inhibited mRNA-level expression of induced inflammatory cytokines produced by HFs via adenosine receptor activation

The synthetic TLR3 activator poly-(I:C) increased the mRNA expression of IL-1β, IL-6 and TNF-α inflammatory cytokines and the chemokine IL-8 in HFs to approx 3-10-fold *in vitro* compared to untreated controls. Simultaneous treatment with low (0,1 μM) concentration CBD resulted in a significant reduction in all cytokines and chemokines tested, in fact, TNF-α and IL-8 levels were virtually normalized. The co-application of CGS 15493, which inhibits all adenosine receptors, prevented the effect of CBD (except for the chemokine IL-8). These results suggest that CBD exerts anti-inflammatory effects by adenosine receptor activation on HFs.

Discussion

The role of TRPV4 in hair cycle regulation

TRPV4 has already been associated with important functions in the skin. In epidermal keratinocytes, TRPV4 contributes to the formation of cell-cell connections at molecular level, which is one of the foundations of mechanical and other barrier functions of the skin. The function of TRPV4 in the hair follicle detailed in my present thesis fits our previous knowledge about the role of TRPV4 in skin. Consistent with previous results from rodents, our results suggest that TRPV4 is predominantly present in HF epithelial cells, mainly in the ORS, which is otherwise continuous with the epidermis that also expresses TRPV4.

The revealed functional presence of TRPV4 fits well with the previously characterized TRP channels (TRPV1 and TRPV3), which also proved to be catagen inducers. The TRPV4 selective agonist reduced hair growth, this was due to a dramatic decrease in proliferation in the hair-forming matrix keratinocytes characteristic of the early catagen phase. Apoptosis of matrix keratinocytes was not necessarily a direct effect, i.e., not a direct consequence of the activation of the TRPV4 channel on MK cells, but probably a well-known phenomenon of catagen transition. However, we showed that TRPV4 activation was also able to directly induce apoptosis in ORSKs, the predominant TRPV4-expressing cell type of HFs. It is likely that the effect of TRPV4 is principally mediated through these cells, as they exhibited the most apparent TRPV4 immunoreactivity, and other effects of TRPV4 activation have been demonstrated in these cells. Based on our studies, TRPV4 activation by a synthetic agonist increased the $[Ca^{2+}]_{ic}$ of ORSKs in a concentration-dependent manner and in the dose range inducing catagen in our *ex vivo* HF organ culture. Our results suggest that TRPV4 is mainly present in the ORS, upon activation, Ca^{2+} influx occurs at a cellular level, which initiates apoptosis. These effects on whole, intact HFs lead to the initiation of regressive

processes, thus inducing a catagen transition, similarly to the effects of TRPV3 stimulation on HFs.

It is also an interesting idea whether TRPV4 does not simply complement the presence of other TRPV receptors expressed on HF since these channels have been known to form heterotetramers. Although, in theory, any of the three TRP channels already described on HF can form a heteromer, there are also results that co-expression of TRPV1-4 ion channels tends to form homotetramers. In epidermal keratinocytes, TRPV4 plays a role in maintaining the barrier through the formation of intercellular connections, and temperature also plays a regulatory role in this process. In addition to the epidermis, TRPV4 regulates the diameter of blood vessels in the skin stimulated by heat stimuli, so it may also play a role in heat regulation. In mouse keratinocytes, the activation threshold temperature of TRPV4 was measured to be 32 ^{o}C, and in our experimental setup, a similar temperature stimulus of 31 ^{o}C was able to induce Ca^{2+} influx on ORSKs. The otherwise effective TRPV4 antagonist HC067047 could only partially prevent Ca^{2+} influx on ORSKs even at relatively high concentrations of 10 μM. Based on these results, TRPV4 contributes to the increase in $[Ca^{2+}]_{IC}$ upon thermal stimulation of ORSKs.

Another question is whether the heat-sensitivity assumed based on our results, might also manifest under *in vivo* conditions. To date, TRPV4 on dermal cells has been reported to be activated by thermal stimulation *in vitro*. Furthermore, it is general characteristic of the kinetics of TRP channels that sustained stimulation with the activating temperature can lead to inactivation of the ion channel since thermal stimulus only shifts the voltage threshold (for both activation and inactivation). It would also mean that TRPV4 is inactive in the skin *in vivo* as it is located in an area that has a temperature close to the (in)activating temperature range.

Changes in the hair cycle and the quantity and quality of hair associated with seasons or temperature change are well documented in the animal kingdom. No

such phenomenon is known in humans, however, changes in temperature around HF may be significant in clinical practice. On one hand, dedicated devices successfully reduce the rate of hair loss associated with chemotherapy by cooling the scalp, and on the other hand, treatment based on the principle of selective photothermolysis (restricted to HFs) provides effective and long-lasting hair removal. The possible involvement of TRP channels in the background of these heat induced phenomena has not been studied so far.

Each of several stimuli (including heat, mechanical deformation, pH) co-occurring during inflammatory processes can activate TRPV4 alone, moreover, they also potentiate each other's effect on TRPV4 activation. Inflammation, as well as multiple inflammatory mediators alone can lead to hair loss or the formation of a catagen phase *in vitro*. It is therefore suspected that TRPV4 also contributes to the hair loss that accompanies inflammation.

Finally, seeking for possible *in vivo* activators of TRPV4, the endocannabinoid anandamide emerges as a potential endogenous ligand. Moreover, HF is able to produce anandamide, which, although also itself is a catagen inducer, exerts its effects through the CB1 receptor.

Thus, our results suggest that TRPV4 fits well among the members of the endocannabinoid-TRP system described so far on human HFs, since activation of both the CB1 metabotropic receptor and TRPV1,3-4 ion channels in human *ex vivo* HF organ culture induces a catagen phase. The similarity of the expression pattern of TRP channels within the HF is remarkable; their expression dominates in the ORS layer, but none have been confirmed in DP cells. However, there are also differences in the effects they mediate; thus, TRPV3 activation of ORSKs also triggered both apoptosis and necrosis, whereas TRPV4 activation, according to our results, caused purely apoptosis. In the future, in addition to pharmacological targeting of TRPV4, it is worth conducting further research to characterize the effects of TRPV4 on HF *in vivo* and to investigate the role of

TRPV4 in the background of possible endogenous activators (e.g., heat, inflammation).

Effects of CBD on biological processes in HF

According to our previous results, CBD exerts promising anti-acne effects on the other member of the PSU, the sebaceous gland, mediated in part by TRPV4 and adenosine receptor activation. The proximity of the two "mini-organs" and their common role in the development of acne and other diseases justifies studying a potentially promising drug in both elements of the PSU. Expression of several of the known receptors for CBD has also recently reported on human HFs. Among these, however, certain receptors may mediate opposite effects, for example, activation of TRPV4 shifts the hair cycle toward a catagen direction whereas adenosine receptor activation propagates anagen phase.

Our results suggest that CBD may exert different or even opposite effects depending on the concentration range it is applied, and this is due to the activation of different receptors at different concentrations. Hair growth was rather enhanced at low concentrations of 0.1 μM (although insignificantly) without any significant effect on hair cycle or matrix keratinocytes' processes (apoptosis / proliferation). Low-concentration CBD treatment also had no effect on Ca^{2+} homeostasis in ORSKs. In this low concentration range up to 1 μM, it appears safe without activating TRPV4, and it has no effect on ORSK viability, as it does not induce apoptosis or necrosis.

However, CBD at high concentrations of 10 μM inhibited hair growth *ex vivo* and the proportion of apoptotic cells in the MK region rose from a negligible few percent to about 15%. Accordingly, high concentration CBD also dramatically increased the proportion of catagen HFs. Importantly, in this concentration range (above 10 μM) it also significantly increases the $[Ca^{2+}]_{ic}$ of ORSKs, and CBD-induced Ca^{2+} influx can be significantly prevented by the simultaneous use of the TRPV4 antagonist HC067047. CBD has a similar effect on the viability of ORSKs

to TRPV4 activation, i.e., it can induce apoptosis, but it is also accompanied by necrosis. CBD at this concentration also inhibits cell proliferation and viability.

CBD at low concentrations, in addition to appearing to be safe, i.e., lacking the TRPV4-mediated effects described above, has also been shown to have anti-inflammatory activity. In our model, we induced an *in vitro* inflammatory environment in HF organ culture by activating the TLR3 receptor, during which we managed to increase the cytokine production of untreated HFs, so that the expression of IL-1, IL-6, IL-8 and TNFα increased 3-10 times. CBD was able to minimize or prevent this inflammatory response when co-administered and presumably exerted this effect by activating adenosine receptors. Our results suggest that CBD, when used at a sufficiently low dose in which TRPV4 is not yet substantially activated, was able to exert an anti-inflammatory effect by stimulating adenosine receptors. This explains why CBD can also insignificantly enhance hair growth at this dose.

Chiefly because of its anti-inflammatory effect, CBD was first studied in acne among skin diseases in a clinical trial. The results of this placebo-controlled, double-blind, randomized study (NCT03573518) have not published yet, however, based on preliminary results, subjects tolerated twice daily topical treatment well. The anti-acne effect of CBD may be due in part to the complex anti-inflammatory, antiproliferative and sebostatic effects on sebaceous glands previously reported by our group. Given that increased proliferation in the ORS layer also plays a role in the development of acne, CBD's antiproliferative and anti-inflammatory effects on ORSKs may also contribute to therapeutic success. The effect of local and systemic CBD treatment is currently being studied in several other skin diseases (NCT04045119l, NCT03824405, NCT03693833). The interest in topical CBD treatment is justified, as anti-inflammatory agents are much needed in the treatment of common, immune-mediated skin diseases. In addition, systemically safe CBD may have an even narrower side effect profile with topical treatment.

Overall, the results obtained from our *in vitro* and *ex vivo* human experiments are consistent with research findings about CBD collected so far, suggesting that CBD remains a promising drug candidate in dermatology. Furthermore, our results also bring us closer to understand the complex mechanisms behind the effects of CBD.

Summary

In the current study we aimed to increase the understanding of the physiological role of the ECS on HFs and we introduced a novel hair cycle regulator of the TRP channel family. Our results suggest that human HFs express functionally active TRPV4, particularly in the ORS layer. In the ORSKs, TRPV4 functions as a Ca^{2+}-channel that can be activated by thermal stimulus *in vitro*. Chemical TRPV4 activation leads to apoptosis on ORSKs, whereas, when targeted on intact human HFs *ex vivo*, TRPV4 activation induces catagen transition along with suppressed hair growth.

Moreover, we tested the effects of CBD on human HFs and characterized the receptors that mediate its effect. We found that CBD-treatment exerted opposite effects depending on the concentration applied. We assume that CBD acts as an agonist on both TRPV4 and adenosine receptors that have opposite effects on the hair cycle. At low concentrations it has minor effects on the hair cycle and enhances hair growth, although statistically insignificantly. Additionally, also at low concentrations, CBD has anti-inflammatory effects mediated by adenosine receptor activation. However, at high concentrations, CBD activates TRPV4 channels, dominating its effects on HFs, leading to decreased hair growth and catagen morphology. On ORSKs, the predominant TRPV4-expressing cells in the HF, CBD induces apoptosis and necrosis mediated by TRPV4 activation and resultant Ca^{2+}-influx.

Our results are in line with earlier scientific reports, but they also deliver new data about the ECS-TRP system's function on HFs. We provided evidence that TRPV4 contributes to the TRPV1-TRPV3 and CB1 driven pro-catagen ECS tone on the hair cycle. Moreover, we characterized CBD's complex effect on hair biology and identified its cellular targets as TRPV4 and adenosine receptors on ORSKs.

APPENDIX

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List of publications related to the dissertation

1. **Szabó, I. L.**, Herczeg-Lisztes, E., Béke, G., Tóth, K. F., Paus, R., Oláh, A., Bíró, T.: The phytocannabinoid (-)-cannabidiol (CBD) operates as a complex, differential modulator of human hair growth: anti-inflammatory submicromolar versus hair growth inhibitory micromolar effects.
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   DOI: http://dx.doi.org/10.1016/j.jid.2019.07.690
   IF: 6.29 (2018)
2. **Szabó, I. L.**, Herczeg-Lisztes, E., Szegedi, A., Nemes, B. Á., Paus, R., Bíró, T., Szöllősi, A. G.: TRPV4 Is Expressed in Human Hair Follicles and Inhibits Hair Growth In Vitro.
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List of other publications

3. Herczeg-Lisztes, E., Tóth, I. B., Bertolini, M., **Szabó, I. L.**, Zákány, N., Oláh, A., Szöllősi, A. G., Paus, R., Bíró, T.: Adenosine promotes human hair growth and inhibits catagen transition in vitro: role of the outer root sheath keratinocytes.
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