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The role of the endocannabinoid system in regulating the biological processes of the skin

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The Examination takes place at the Department of Medical Chemistry, Faculty of Medicine, University of Debrecen
at 11 am, on September 17, 2014

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen
at 1 pm, on September 17, 2014

Introduction

The endocannabinoid system (ECS)

The Endocannabinoid System (ECS) was first discovered when research focused on the distinct natural compounds (called cannabinoids) responsible for the effects attributed to the *Cannabis sativa* plant. It was the isolation of the main psychoactive component, (−)-trans-Δ9-tetrahydrocannabinol (THC), in 1964 by Mechaoulam's group, that truly sparked the study of cannabinoid chemistry. The specific receptor activated by cannabinoids was identified in 1988 from rat brain and subsequently dubbed CB1. The next larger milestone was the discovery a second cannabinoid receptor (dubbed CB2) from HL-60 cells and identified in macrophages residing in the marginal zone of the spleen, and ultimately cloned from a mouse splenocyte cDNA library. Although these classical cannabinoid receptors were originally thought to be expressed in specific tissues (the central nervous system for CB1 and non-neuronal tissues for CB2), it is now apparent that both receptors are widely distributed in many cell types.

The cloning of the first cannabinoid receptors triggered a search for endogenously occurring counterparts of plant-derived cannabinoids. The first two such endocannabinoids, both derivatives of arachidonic acid, were isolated a mere three years apart; N-arachidonoyl ethanolamine (anandamide or AEA) and 2-arachidonoylglycerol (2-AG). Although both their general structure and functions are similar, the biochemical steps of their synthesis and degradation, as well as their receptor affinity differ widely. AEA is mainly thought to be generated via N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD) while 2-AG is mainly synthesized by subsequent steps of phospholipase C and diacylglycerol-lipase (DAGL) α and β. The inactivation of both endocannabinoids is also dependent on separate degrading enzymes, namely fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase

(MAGL) for AEA and 2-AG, respectively. Since both of these prototypic endocannabinoids can bind to both CB1 and CB2 receptors (albeit with different affinities), early studies regarded them as mutually exchangeable in the modulation of synaptic signaling. More than two decades worth of observations have provided conclusive evidence that AEA and 2-AG are by far not similar in their functioning.

Although AEA and 2-AG are the two most extensively researched endocannabinoids, other endogenous ligands have been found to share some cannabimimetic actions. As such, the term “endocannabinoid” encompasses a steadily growing family of molecules (i.e. palmitoylethanolamide [PEA], oleamide, 2-arachidonoylglycerol ether, virodhamine and lysophosphatidyl inositol [LPI]) which can in no way be considered complete. Many of these novel endocannabinoids do not only activate classical cannabinoid receptors but numerous others as well, thereby effectively expanding the receptor-arsenal of the ECS to include novel metabotropic (e.g. GPR18, GPR55, GPR119), ionotropic (e.g. thermosensitive transient receptor potential [TRP] channels) and nuclear (e.g. peroxisome proliferator-activated receptors [PPAR]) receptors.

Novel metabotropic receptors GPR18 and GPR119 were identified in broad expression studies of GPCRs and a bioinformatic search of the human genome database respectively. Although neither receptor shows significant sequence homology with classical cannabinoid receptors, recent results on the function of these receptors have supported the hypothesis that they are activated by cannabinoid ligands. GPR18, which is widely expressed on many tissues, has been presented as the abnormal cannabidiol (CBD) receptor and has previously been implicated in the modulation of microglial, endothelial and glioma cell migration, and a selection of cardiovascular responses. GPR119 was assigned to the receptor cluster encompassing the cannabinoid receptors, and has been shown to take part in metabolic homeostasis and in the control of energy balance

via the regulation of incretin-dependent insulin release and the secretion of glucagon-like peptide 1 from enteroendocrine cells.

GPR55, discovered in silico and cloned in 1999, binds multiple cannabinoids with high affinity, including synthetic CB1 and CB2 agonists HU210 and JWH015, endocannabinoids 2-AG and LPI, CB1 antagonists AM251 and rimonabant as well as phytocannabinoids such as CBD and THC. Since the pharmacology of the receptor is complex, the true endogenous ligand has not been conclusively identified. In spite of this ambiguity, numerous (patho)physiological processes have been linked to GPR55. These include putative roles in the cardiovascular system, in nociception, in inflammation, in the regulation of energy metabolism and in bone metabolism. GPR55 is also implicated the regulation of cellular proliferation and cancer formation, since its expression is augmented in different neuronal and peripheral cells, with mRNA and protein levels appeared to correlated with tumor aggressiveness.

Although endocannabinoid ligands have classically and most extensively been described to activate metabotropic receptors, the promiscuity of the ECS also encompasses ionotropic receptors; namely the thermosensitive TRP channels. TRP channels represent a superfamily of (mostly) non-selective cation channels further classified into – as of the current writing – six subfamilies: canonical (TRPC), vanilloid (TRPV), melastatin (TRPM), polycystin (TRPP), mucolipin (TRPML) and ankyrin (TRPA).

The link between TRP channels and the ECS was first suggested based on functional and anatomical similarities between TRPV1 and proteins of the ECS. Indeed, the first endogenous ligand of cannabinoid receptors, AEA, has the distinction of being the first endogenous ligand of TRPV1 as well. 2-AG, the second most studied endocannabinoid has negligible effect on TRP channels; however, certain effects such as the stimulation of Ca^{2+} influx in microvascular endothelial cells and the anti-proliferative action on C6 glioma cells were attenuated by capsazepine (CPZ), a TRPV1 antagonist. Whether this effect can

be attributed to the lack of selectivity of CPZ or to the conversion of 2-AG to diacylglycerols which might activate TRPV1 has not been elucidated as of this writing.

Nuclear receptors, i.e. the peroxisome proliferator-activated receptors (PPAR), a superfamily of nuclear hormone receptors, have also been shown to respond to cannabinoids. Two PPARs have been found to be activated by cannabinoids: PPARα and PPARγ. A number of cannabinoids show appreciable receptor binding affinity, such as THC and AEA. In behavioral studies, the inhibition of FAAH – and the concomitant increase in AEA and PEA – resulted in an anti-inflammatory effect which could be abolished by the application of a PPARα antagonist

The ECS in the skin

Classically, the main function of the skin has been considered to form a passive physico-chemical barrier against environmental challenges. However, more recent research has highlighted the complexity of the skin and its' adnexal components (i.e. hair follicles, sebaceous and sweat glands), thereby proving that it functions as an active neuro-immuno-endocrine organ. In the past decade, a plethora of research (not in small part from our own workgroup) has highlighted the largest organ of the body, the skin, as an important site of action of the ECS.

Classical ECS receptors (CB1 and CB2) are differentially expressed in the human epidermis, with higher expression found in the more differentiated (i.e.: the granular and spinous) layers, which suggested their involvement in the regulation of differentiation and proliferation of these cells. Indeed, both phytocannabinoids and synthetic CB agonists inhibited the proliferation of cultured transformed human epidermal keratinocytes, although this effect proved to be independent of CB1 and CB2. However, our workgroup found that AEA markedly and dose-dependently caused cell death in cultured human

keratinocytes, while inhibiting proliferation and inducing apoptosis in situ, which actions were dependent on both CB1 and TRPV1 expressed by these cells through a sequential CB1→TRPV1→Ca^{2+} influx signaling pathway.

AEA has also been shown to regulate human epidermal differentiation, since locally produced AEA causes the transcriptional downregulation of keratin 1, keratin 5, involucrin and transglutaminase-5. In terms of skin barrier function, CB1 and CB2 knockout mice display markedly different rates of barrier recovery after tape stripping; CB1 deficient mice exhibited delayed recovery, while this was increased in CB2 knockout mice, mainly due to increased lamellar body secretion.

Ionotropic cannabinoid receptors (i.e. thermo-sensitive TRP channels), as Ca^{2+} permeable channels, significantly modulate cellular Ca^{2+} homeostasis which is a key regulatory process that affects proliferation, differentiation and mediator production of various skin cells. On cultured human keratinocytes, stimulation with capsaicin or heat treatment induced TRPV1-dependent Ca^{2+} influx (since the concomitant application of the TRPV1 antagonist CPZ could effectively block these changes), proving that the channel is functionally expressed on these cells. This elevation of intracellular Ca^{2+} decreased proliferation of cultured normal human epidermal keratinocytes (NHEKs) and induced apoptosis. It is not surprising therefore that TRPV1 activation delayed barrier recovery after tape stripping, which effect could also be blocked by CPZ.

Activation of TRPV4, another thermosensitive channel activated by cannabinoids also accelerates barrier recovery in mice. A similar effect was also observed in relation to cold-sensitive channels (TRPA1 and TRPM8). TRPA1 also influences cutaneous inflammation, since its activation enhanced ear swelling response and dendritic cell migration in a contact hypersensitivity model, while in vitro it evoked the production of interleukin IL-1α and IL-1β.

The ECS (and most especially CB2) has been conclusively proven to influence many aspects of both innate and adaptive immunity; overall, the ECS

promotes mainly anti-inflammatory processes. On the immune cells of the skin, data is relatively sparse in relation to the ECS. Our own workgroup has shown however that monocyte-derived human dendritic cells (DC) not only express TRPV1 through the differentiation of monocytes to immature DCs (iDC), but the activation of TRPV1 channels expressed on iDCs and mature DCs (mDC) could be activated by the application of capsaicin, arguing for the functional expression of these proteins. Interestingly, the long-term application of capsaicin (5 days) did not induce either the differentiation of monocytes, or the activation of iDCs, in contrast to previous data reported on mice. Likewise, capsaicin did not significantly alter the viable cell number nor did it induce cell death of any form even at relatively high concentrations. One of the most important functions of iDC is the phagocytosis of foreign material, which was also inhibited by both long- and short-term capsaicin treatment in a TRPV1-dependent manner.

Goals

The overview presented above shows the ECS is not only complex in its heterogeneity and interactions with signaling systems previously thought to be unrelated to cannabinoids, but it also influences an ever-expanding array of physiological processes in practically all organs of the body. The overarching research goal of our workgroup is the investigation of the role of the ECS in the physiological processes of the skin. As part of our investigations, our research area presented in this thesis, on one hand, focused on elucidating the expression and putative functionality of the ECS on eccrine sweat gland cells, since the ECS was shown to have important regulatory roles on the biological processes on sebaceous glands, the other exocrine organ of the skin. On the other hand, we aimed at further elucidating the role of thermosensitive TRP channels on dendritic cells; since TRPV1 was shown to take part in the transduction of heat shock signaling in keratinocytes we aimed at determining whether it plays a similar role on iDCs.

Specifically, on eccrine sweat gland-derived NCL-SG3 epithelial cells, we aimed at investigating:

- The effect of endocannabinoids on the
    - Viability, apoptosis and necrosis of the cells
    - Differentiation of the cells
    - Secretory activity of the cells
- The expression of members of the ECS
- The signaling pathways involved in putative endocannabinoid effects

On human monocyte-derived DCs, we aimed at assessing:

- The expression of thermosensitive TRP channels
- The effect of heat shock on endocytotic activity of iDCs
- The involvement of thermo-TRP channels in mediating the putative heat shock effects

Materials and methods

NCL-SG3 cell cultures

Human eccrine sweat gland-derived NCL-SG3 epithelial cells were cultured in William's Medium E medium supplemented with 5 % fetal bovine serum, 10 μg/ml insulin-transferrin-selenium mixture, 20 ng/ml epidermal growth factor, 2 mM L-glutamine, 10 ng/ml hydrocortisone, and antibiotic mixture. Cells were passaged once they reached 80-90% confluence to avoid confluence-induced differentiation.

DC cultures

Thrombocyte-free buffy coats were centrifuged on Ficoll gradient to separate the monomorphonuclear cells. Monocytes were subsequently isolated by immunomagnetic cell separation using anti-CD14-conjugated microbeads. DC differentiation (which resulted in iDCs) was induced by supplementing AIMV medium with GM-CSF and IL-4 on the first and second days, and the cells were cultured for 5 days. mDCs were generated by exchanging the culturing medium of iDCs with AIMV supplemented with a "pro-inflammatory cytokine cocktail" containing 80 ng/ml GM-CSF, 10 ng/ml TNF-α, 5 ng/ml IL-1β, 20 ng/ml IL-6, and 1 μg/ml PGE2 for one additional day. Cells were cultured at 37 °C, in a humidified environment containing 5% CO_2. All experiments were performed from a minimum of three independent donors.

Heat shock treatment and determination of endocytotic activity

Heat shocked DCs were incubated at 43°C for 1hr, while control cells were kept at 37°C. Endocytotic activity was then measured by the internalization of FITC-labeled dextran (1 mg/ml FITC-dextran at 37°C for 1 hr after heat shock treatment). Afterwards, the cells were washed three times with ice-cold phosphate buffered saline (PBS), and then stored on ice until the fluorescence intensity was measured by a FACScan flow cytometer. When applicable, DCs

were pre-incubated with CPZ or HC 067047 (TRPV4 antagonist) for 15 minutes before heat shock, or with the appropriate vehicle control (Dimethyl sulfoxide for both compounds).

Determination of cytotoxicity (necrosis)

Necrotic cell death was determined by measuring the glucose-6-phophate-dehydrogenase (G6PD) release, or the intensity of Sytox Green staining, according to the manufacturer's protocol. Cells (10,000 cells/well in the case of NCL-SG3 cells and 200,000 cells/well in the case of monocytes/iDCs) were cultured in 96-well black-well/clear-bottom plates in quadruplicates and treated with various compounds for 24-48 hrs (NCL-SG3 cells) or subjected to heat shock as described above. The fluorescence emission was monitored by a Flexstation 3 fluorescent image plate reader (FLIPR) at 545 nm excitation and 590 nm emission wavelengths for G6PD release and 490 nm excitation and 520 nm emission for Sytox Green.

Determination of apoptosis

Apoptosis of iDCs was detected by using the "Mitochondrial Membrane Potential Apoptosis Kit with Mitotracker™ Red & Annexin-V Alexa Fluor® 488 – for Flow Cytometry" according to the experimental protocol supplied by the manufacturer. Cells were subjected to heat shock treatment as described previously or left at 37°C for 1 hr. Sequential double labeling was then performed by the MitoTracker Red/Alexa Fluor® 488 annexin-V Reagents and the cells were analyzed by a FACScan flow cytometer. Emissions of the dyes were measured at 530 nm and 585 nm, respectively.

A different combination of techniques was used for the determination of apoptosis in NCL-SG3 cells. Mitochondrial membrane potential of NCL-SG3 cells was determined using a MitoProbe™ DiIC1(5) Assay Kit, while the activation of pro-apoptotic caspases were determined using a Poly Caspases

Detection Kit. Cells (10,000 cells/well) were cultured in 96-well black-well/clear-bottom plates in quadruplicates and were treated with various compounds for the time indicated. The cells were subsequently stained according to the supplied protocol, and fluorescence was measured at 630 nm excitation and 670 nm emission and 490 nm excitation and 530 nm emission wavelengths, respectively, using a FLIPR.

Determination of viable cell numbers

Cells were cultured in 96-well plates and the number of viable cells was determined by using an MTT based colorimetric assay. Supernatants were removed and cells were then incubated with MTT working solution. The reaction product formazan crystals were disolved in HCl diluted in isopropanol, and concentration was determined colorimetrically at 550 nm. The absorbance was proportional to the number of living cells.

Determination of proliferation

Proliferation was assessed by the CyQuant Cell Proliferation Assay Kit according to the manufacturer's protocol. Cells (10,000 cells/well) were cultured in 96-well black-well/clear-bottom plates in quadruplicates and were treated with various compounds for the time indicated. After appropriate staining, fluorescence was measured at 490 nm excitation and 520 nm emission wavelengths using a FLIPR.

Determination of endocannabinoid levels

Cultured NCL-SG3 cells were homogenized in 0.5 ml of an ice-cold solution of methanol: Tris buffer (50 mM, pH 8) 1:1 containing 7 ng of $^{2}H_{4}$-AEA. After chemical processing and reconstitution analysis was performed by liquid chromatography/in line mass spectrometry. Liquid chromatographic separation of endocannabinoids was achieved using a guard column and

analytical column at 32 ºC. The MSD (model LS) was set for atmospheric pressure chemical ionization (APCI) and selected-ion-monitoring (SIM) to monitor ions m/z 348 for AEA, 352 for $^{2}H_{4}$-AEA, and 379 for 2-AG. The spray chamber settings were: vaporizer 400ºC, gas temperature 350ºC, drying gas 5.0 l/min, and nitrogen was used as the nebulizing gas with a pressure of 60 psig. Calibration curves were produced using synthetic AEA and 2-AG. The amounts of AEA and 2-AG in the samples were determined using inverse linear regression of standard curves.

Quantitative determination of intracellular lipids

Cells (10,000 cells/well) were cultured in 96-well black-well/clear-bottom plates in quadruplicates and were treated with compounds for 24 hrs. Subsequently, supernatants were discarded and 100 µl of a 1 µg/ml Nile Red solution in PBS was added to each well. Fluorescence was measured on a FLIPR and results are expressed as percentages of the relative fluorescence units (RFU) in comparison with the controls using 485 nm excitation and 565 nm emission wavelengths for neutral (mostly cytoplasmic) lipids, and 540 nm excitation and 620 nm emission wavelengths for polar (mostly membrane) lipids.

Immunocytochemistry

Monocytes were seeded onto glass coverslips and iDCs were generated as described above. iDCs were fixed with acetone for five minutes and permeabilized with PBS containing 0.1% Triton-X-100. Cells were subsequently washed with PBS and non-specific binding sites were blocked with PBS containing 1% bovine serum albumin (BSA). Immunolabeling was performed by incubating the cells with polyclonal rabbit antibodies against TRPV1, TRPV2, TRPV3, TRPV4, TRPA1 and TRPM8 (1:200 dilution) for 60 minutes. Coverslips were subsequently washed three times in PBS and further incubated with a FITC-conjugated secondary antibody (1:200) for 60 minutes. Cell nuclei

were stained with DAPI after three final washes with PBS and visualized using a Nikon Eclipse E600 fluorescent microscope.

Western blotting

Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to BioBond nitrocellulose membranes, and then probed with rabbit primary antibodies against CB1, CB2 (both 1:200), TRPV1, TRPV2 and TRPV4 (all used at 1:500 dilution). A horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:1000) was used as a secondary antibody, and the immunoreactive bands were visualized by a SuperSignal® West Pico Chemiluminescent Substrate enhanced chemiluminescence kit using LAS-3000 Intelligent Dark Box. To assess equal loading, membranes were re-probed with an anti-cytochrome-C (1:50) or anti-beta-actin antibody (1:1000) and visualized as described above. Where appropriate, immunoblots were subjected to densitometric analysis using the Image Pro Plus 4.5.0 software.

Quantitative real-time PCR (Q-PCR)

Q-PCR was performed on an ABI Prism 7000 sequence detection system by using the 5′ nuclease assay. Total RNA was isolated using TRIzol. One μg of total RNA were then reverse-transcribed into cDNA by using 15 U of AMV reverse transcriptase (RT) and 0.025 μg/μl random primers. PCR amplification was performed by using the TaqMan primers and probes using the TaqMan universal PCR master mix protocol.

Gene silencing using small interfering RNA (RNAi) probes

iDCs or NCL-SG3 cells at 50-70% confluence were transfected on the second day of differentiation or culturing after passaging with specific Stealth RNAi oligonucleotides (40 nM) against TRPV channels or classical cannabinoid receptors using Lipofectamine 2000 Transfection Reagent. For controls, RNAi Negative Control Duplexes (scrambled RNAi) were employed. The efficacy of RNAi-driven ‘‘knockdown’’ was evaluated daily (in the case of NCL-SG3 cells) or on day 3 after transfection (for iDCs) by Western blotting as described above.

Optical density was normalized to β-actin (1:1000 dilution) and expressed relative to cells transfected with scrambled RNAi.

Patch-clamp measurements

The standard whole-cell patch-clamp techniques were employed in voltage-clamp configuration. The temperature of the EC solution perfusing the DCs was maintained at a constant level of either ~37°C or ~45°C. The bath solution was warmed up to ~37°C using a custom-made Petri dish. Thermal stimuli were applied using a preheated bath solution and temperature was monitored using a thermistor placed close to the cell. Bath perfusion around the measured cell was achieved using a gravity-flow perfusion system. RR, CPZ and HC 067047 were applied by switching the perfusion system to a preheated bath solution containing the relevant antagonists.

Immunoprecipitation

Cells were harvested into 1 ml of radioimmunoprecipitation assay (RIPA) buffer and were disrupted by sonication on ice. Immunoprecipitation was carried out on prepared samples with 5 μl of anti-TRPV2 antibody by rotating the samples overnight at 4 °C. The samples were spun at 15,000×g at 4 °C for 5 min, the pellet was washed three times with RIPA buffer, and then resuspended in SDS-PAGE sample buffer and boiled for 10 min at 100 °C. All samples were then subjected to Western blotting as described above.

Statistical analysis

When applicable, data were analyzed using a two-tailed un-paired t-test and $P<0.05$ values were regarded as significant differences.

Results

1. Role of the ECS in sweat gland biology

Endocannabinoids cause mainly apoptotic cell death in sweat gland cells

Our initial question in relation to sweat gland cells was whether classical endocannabinoids, AEA and 2-AG, effect the life cycle of the NCL-SG3 sweat gland cell line. Using colorimetric MTT and fluorimetric CyQuant assays, we found that both endocannabinoids dose-dependently suppressed the viable cell number and proliferation of NCL-SG3 cells. Based on quantitative fluorimetric determinations, both endocannabinoids significantly decreased mitochondrial membrane potential and induced the activation of pro-apoptotic caspases, hallmarks of apoptosis. In addition, higher concentrations of 2-AG significantly increased the release of G6PD and Sytox Green accumulation to the cells, two complementary indicators of necrosis/cytotoxicity. These findings suggested that the endocannabinoids suppressed cellular growth and induced chiefly apoptosis-driven cell death of human sweat gland cells.

Endocannabinoids modulate expressions of cytoskeletal proteins and lipid synthesis of NCL-SG3 cells

Since most epithelial cells initiate their differentiation program with the onset of apoptosis, we next wished to investigate whether endocannabinoids also take part in the regulation of this process on sweat gland cells. Since the exact details of the differentiation processes have not been investigated on cultured sweat gland cells, we analyzed the expression of various epithelial “differentiation markers” such as cytokeratins (CK) as well as of involucrin, filaggrin, and loricrin in NCL-SG3 cells. Interestingly, expressions of certain CKs, namely CK7, 14, 18 and 19, were the highest in pre-confluent proliferating cultures, while CK8, involucrin filaggrin and loricrin were predominant in post-confluent, and presumably more differentiated cells.

We next investigated the effects of AEA and 2-AG on the expression of these markers. Pre-confluent (30-40%) NCL-SG3 cells were treated with AEA and 2-AG (10 μM each) for 48 hrs and the levels of the above markers were analyzed by quantitative "real-time" QPCR. The expression of post-confluent markers (CK8, involucrin, filaggrin and loricrin) were significantly and differentially increased by the two endocannabinoids. The expression of certain markers of proliferating cells were concomitantly suppressed upon endocannabinoid treatment (CK7 by 2-AG; CK14 by AEA and 2-AG).

Endocannabinoids have previously been shown to markedly increase the lipid synthesis of cultured human sebocytes. Since sweat gland epithelial cells were shown to synthesize a wide-array of lipids, we also assessed whether endocannabinoids have a similar effect on the lipid synthesis of NCL-SG3 cells. As measured by semi-quantitative Oil Red-O histochemistry and by quantitative Nile Red-based fluorimetry, both endocannabinoids (as early as 24 hrs after treatment) markedly and dose-dependently elevated neutral (but not polar) lipid synthesis of the cells. Neutral lipids reflect the intracellularly stored, "de novo" synthesized lipid pool; as such, our data suggest that endocannabinoid treatment may exert a profound role in the regulation of secretory activity of human eccrine sweat gland cells by modifying the composition of produced sweat.

Multiple members of the ECS are expressed by NCL-SG3 cells

AEA and 2-AG are both known to activate metabotropic and ionotropic cannabinoid receptors. To determine which of these possible targets are present on sweat gland cells, we performed QPCR and Western blot analysis. CB1 and CB2 are expressed on both the mRNA and protein level; CB1 expression was the highest in the post-confluent cultures, with the expression increasing during culturing, while CB2 levels were highest in pre-confluent cultures, and its expression decreased with culture time. These data suggest that cannabinoids

might play a role in the regulation of growth and differentiation of human sweat gland-derived cells.

Since numerous skin cells have been shown to produce endocannabinoids (see above), with the help of our collaborators we also investigated whether sweat gland epithelial cells could also be considered a source of these lipid mediators. Mass spectrometry analysis revealed that NCL-SG3 sweat gland cells produce the prototypic endocannabinoids AEA and 2-AG; however, at a much lower concentrations than those reported in other skin cells (AEA, 15 fmol/10^6 cells; 2-AG, 0.2 pmol/10^6 cells in NCL-SG3 cells compared to AEA, 160 fmol/10^6 cells; 2-AG, 4.2 pmol/10^6 cells in human SZ95 sebocytes).

Based on these data we next examined the expression of the enzymatic machinery required for their synthesis and degradation. Using QPCR we were able to determine that not only are the synthesizing (NAPE-PLD and DAGLα and β) and degrading enzymes (FAAH and MAGL) present on the cells, but their expression level fluctuates in parallel with the confluence of the cells; this further suggests that there is a possible role for the ECS in the regulation of growth and most probably differentiation of these cells.

Endocannabinoid effects are not mediated by classical metabotropic receptors or TRP channels

After demonstrating that endocannabinoids (i) cause apoptotic cell death; (ii) increase the expression of differentiation-associated markers; and (iii) modulate the composition of secreted sweat, we next wished to elucidate the putative involvement of the cannabinoid receptors expressed by these cells. To assess the role of classical metabotropic cannabinoid receptors CB1 and CB2, we employed various antagonists and the RNAi technique on the endocannabinoid effects on cellular growth, survival and secretory activities. Inhibitors of CB1 and CB2 receptors had no effect when applied independently of AEA or 2-AG. Likewise, CB1 and CB2 knockdown by RNAi technology

could not prevent the growth-inhibitory and differentiation-promoting cellular actions of the tested endocannabinoids. These results argue that CB1 or CB2 coupled intracellular signaling mechanisms most probably do not participate in mediating the effects of endocannabinoids on human sweat gland epithelial cells. Since sweat gland cells also express TRP channels that have been implicated as ionotropic cannabinoid receptors, we next investigated whether Ca^{2+}-influx is involved in the action of AEA and 2-AG. Neither endocannabinoid caused alteration in intracellular calcium concentration; moreover, neither the "universal" TRP channel antagonist Ruthenium Red nor the suppression of extracellular Ca^{2+} had any effect on the cellular growth suppression or lipid induction observed upon endocannabinoid treatment. Taken together, these findings suggest that the effects of cannabinoids are not relegated through either metabotropic or ionotropic cannabinoid receptors, identified by us in human sweat gland epithelial cells.

Endocannabinoids selectively stimulate the MAPK pathway in NCL-SG3 cells

Although our efforts to determine the receptor(s) activated by endocannabinoids proved sadly fruitless, we also aimed at identifying the putative intracellular signaling pathways involved in the transduction of their effects. Since cannabinoids are known to activate a wide range of signaling pathways, we investigated the potential involvement of the MAPK, protein kinase C (PKC) isoenzymes and phosphatidylinositide 3-kinase (PI-3K). Of the tested pharmacological inhibitors only the MAPK inhibitor, PD098059, prevented the effects of both AEA and 2-AG. Furthermore, the endocannabinoids also induced the transient phosphorylation of the MAPK Erk1/2 (p42/44), which effect was also abrogated by the application of the aforementioned antagonist. These findings collectively argued for the crucial involvement of the MAPK pathway in mediating the actions of endocannabinoids in human sweat gland epithelial cells.

2. Role of TRP channels in DC biology

DCs express various heat-sensitive TRP channels

In the first step of these experiments, we aimed at elucidating the putative involvement of thermosensitive TRP channels in the transduction of heat shock on human monocyte-derived dendritic cells. We identified the expression of thermosensitive TRP channels on monocytes, iDCs and mDCs dendritic cells using QCPR. We found that all three cell types express TRPV1, TRPV2 and TRPV4 at the mRNA level, with the expression of all three increasing concomitantly with differentiation. The protein level of these channels was also verified by Western blotting and (in the case of iDCs) with immunocytochemistry. Interestingly, the expression of TRPV3, TRPM8 and TRPA1 was not identified on any of the tested cells by these methods.

Heat shock decreases endocytosis in a TRPV1-independent manner

To determine whether heat shock has similar effects to TRPV1 activation by capsaicin on human monocyte-derived DCs, we investigated its effect on one of the most important functions of iDCs, namely their endocytotic activity. We found that a short heat shock (43ºC for 1 hr) decreased the endocytosis of iDCs whereas, at the same time, it did not induce significant necrotic or apoptotic cell death; this echoes our previous data regarding the activity of TRPV1 on DCs. In spite of the fact that the above temperature range is appropriate for the activation of TRPV1, and, moreover, previous reports have implicated TRPV1 in the transduction of heat shock effects on keratinocytes, antagonism of TRPV1 by CPZ was unable to abrogate the suppression of endocytotic activity. Likewise, RNAi mediated knockdown of TRPV1, albeit efficacious, did not protect DCs from the effect of heat shock. The application of a TRPV4 antagonist, HC 067047, and the RNAi mediated silencing of TRPV4 also failed to influence the effect of heat shock. RNAi mediated knockdown of TRPV2, on the other hand, significantly prevented the action of heat shock (due to the lack of commercially

available, highly selective TRPV2 antagonists, we were unable to perform pharmacological experiments).

Heat shock induces membrane currents which, most probably, are mediated by TRPV2

Since TRPV2 channels seems to be instrumental in transducing the effects of heat shock on iDCs, we next wished to examine the functionality of these molecules using patch-clamp experiments. Using a self-made thermostable patch-clamp setup, we found that heating the cells above 43°C induced a robust membrane current. This current could be reversibly antagonized by the application of the general TRPV antagonist RR, showing that the TRP channels described above could be responsible for the observed currents. The combined pharmacological antagonism of TRPV1 and TPRV4 did not significantly change the observed current. However, of great importance, the RNAi mediated knockdown of TRPV2 completely abolished the heat-induced current, which points to its key role in the formation of the putative heat-activated channel.

TRPV2 forms heteromeric complexes with TRPV1 and TRPV4

The finding that TRPV2 is responsible for the heat activated current is surprising in the sense that this channel is reportedly activated at higher temperatures. However, recent findings on heterologous expression systems that express multiple TRP isoforms may explain how this is possible. In the study mentioned above Cheng et al. (2007) found that TRP channels may also form heteromers, and that these heteromers show intermediate gating properties. Based on these intriguing findings, using immunoprecipitation, we aimed at determining whether the TRPV proteins on iDCs also formed common complexes. We found that the TRPV2-precipitate also stains positively for TRPV1 and TRPV4, which supports the idea that TRPV2-TRPV1 and TRPV2-TRPV4 heteromeric complexes are present in human DCs.

Discussion

As detailed in the Introduction, the ECS has recently emerged as one of the most important signaling systems which is fundamentally involved in the homeostatic regulatory processes of practically all organ-systems of the human body. With respect to the skin, the largest neuro-endocrine organ of the organism, we and others have recently shown that the ECS, via locally produced endocannabinoids and by activating versatile receptor-mediated signal transduction pathways, plays a crucial role in controlling epidermal and adnexal growth, cell fate and survival, differentiation, lipogenesis, and inflammatory/immune processes.

The ECS is involved in the regulation of human sweat gland biology

As part of our quest to obtain a deeper insight to the functionality of the cutaneous ECS, in the first part of our studies, we aimed at examining the effects of classical endocannabinoids AEA and 2-AG on the human sweat gland-derived NCL-SG3 cell line. Our results prove that both these mediators inhibit proliferation, induce cell death, and up-regulate secretory activity (lipid synthesis) of these cells. These data support the concept that human sweat glands may also function as novel targets for endocannabinoids, as important members of the skin neuroendocrine regulatory network.

Since the differentiation program of these cells has not been extensively detailed, we investigated the expression of various cytoskeletal proteins (CKs, involucrin, filaggrin, loricrin) which are expressed in human sweat gland cells and are also known to be involved in the differentiation of certain skin cells such as the keratinocytes of the epidermis and the hair follicles. In these experiments, we presented the first evidence that levels of these differentiation markers in cultured human sweat gland epithelial cells are dependent on the proliferating state of these cells (i.e. proliferation vs. high cell density-induced growth arrest).

We also found that there are very close similarities between the expression pattern of some of these markers to that described in human epidermal keratinocytes; namely the expression of involucrin, loricrin and filaggrin were highest in post-confluent (and more differentiated) cultures.

Once we have determined which markers might be useful to track the differentiation state of sweat gland epithelial cells, we aimed at determining whether endocannabinoid treatment had any effect on these proteins. AEA and 2-AG markedly increased the expression of the aforementioned post-confluent markers while concomitantly suppressing the expression of certain markers expressed dominantly in proliferating cells. These data point to the involvement of endocannabinoids in the regulation of cell growth and death in human sweat gland cells, while at the same time promoting their differentiation process.

Interestingly, these effects were independent of classical metabotropic receptors CB1 and CB2, since neither pharmacological antagonists nor RNAi-mediated silencing of these receptors was capable of influencing the effects of endocannabinoids. We have also shown that NCL-SG3 cells express ionotropic cannabinoid receptors (TRP channels); however, AEA and 2-AG were unable to elevate the intracellular calcium concentration. As such, our efforts to find the receptor activated by these endocannabinoids was sadly unfruitful; however, we determined that AEA and 2-AG selectively stimulated the MAPK pathway (while having no effect on the PI-3K and PKC secondary messenger pathways).

Our workgroup has previously shown that endocannabinoids inhibit hair shaft elongation through CB1 and promote lipid synthesis and apoptosis on human sebaceous gland-derived sebocytes via CB2 receptors. We may now add sweat gland cells as a new target of endocannabinoids, since AEA and 2-AG inhibit proliferation, induce cell death, and stimulate lipid synthesis and differentiation of human sweat gland epithelial cells by activating non-CB1/CB2-coupled signal transduction pathways. The actions ascribed to the ECS in lieu of the above results compared with previously published data on

other skin appendages (i.e. hair follicles and sebaceous glands) highlight the existence and importance of cell type specific and (most probably) receptor-selective regulatory endocannabinoid effects in the human skin.

TRPV2 is involved in mediating the effects of heat-shock in human dendritic cells

In the other part of our studies, we showed that of the thermosensitive TRP channels TRPV1, TRPV2 and TRPV4 are expressed on monocytes, iDCs and mDCs. We furthermore found that a short heat shock challenge (43°C for 1 hr) decreased the endocytotic activity of iDCs, without causing necrotic or apoptotic cell death. Interestingly, although TRPV1 channels were shown to be activated on human keratinocytes by heat shock, on iDCs only the RNAi-mediated silencing TRPV2 was capable of abrogating the effect of heat shock, while neither pharmacological, nor molecular suppression of TRPV1 and TRPV4 had any such effect. Likewise, the heat shock-induced robust membrane currents were selectively and markedly inhibited by TRPV2 “silencing” (whereas modulation of TRPV1 and TRPV4 activities, again, had no effect).

The above data strongly implicate that the cellular effects of heat shock on human DCs are mediated by TRPV2 ion channels. TRPV2 has been shown to have multiple functions on immune cells. Indeed, on the human mast cell line HMC-1, TRPV2 has been shown to mediate the effects of mechanical, heat and red laser-light stimulation to induce degranulation. In murine macrophages, TRPV2 has been found to be instrumental for proper phagocytosis, particle binding, and bacterial lipopolysaccharide induced cytokine production, as well as in podosome assembly. In rats, TRPV2 has also been described on macrophages, Langerhans cells and DCs, although we lack functional data in this species. It appears, therefore, that TRPV2 ion channels are key players in processing the cellular actions of harmful signals on multiple immune cells.

There are multiple explanations as to how TRPV2, which is reported to be activated by temperatures higher than 52°C, mediates the actions of a much lower, 43°C heat challenge. First of all the temperature-gating properties of TRPV2 were originally described on sensory neurons and in heterologous systems (where host cells ectopically expressed the channels). Since we lack information on the related biophysical properties of the naïve TRPV2 channels found on non-neuronal cells, we cannot exclude the possibility that it is different from those found on sensory neurons; hence, the DC-expressed TRPV2 can be activated at somewhat lower temperatures than its sensory neuron-localized counterpart. Actually, differential sensitivities/affinities for various pharmacological agonists of TRPV1 expressed by neurons and non-neuronal cells have already been reported. Another interesting data is that, in newer studies with heterologous expression systems, TRPV channels have been shown to form heteromers which show intermediate temperature-gating properties. Indeed, we have shown that immunoprecipitates of TRPV2 also stain positively for TRPV1 and TRPV4, which suggests that TRPV2-TRPV1 and TRPV2-TRPV4 heteromerization may take place in human DCs. Nonetheless, it is apparent that TRPV2 is the most “active” component since i) it is the most abundant TRPV channel expressed by human DCs; ii) its molecular suppression fully prevented the actions of heat shock.

Taken together, although further biophysical and biochemical studies are invited to uncover the molecular assembly and gating properties of thermo-TRPs expressed by human DCs, our presented results strongly argue for the central role of TRPV2 in mediating the cellular action of heat shock on these cells.

Perspectives

Evidently, based on our presented current and previous pre-clinical findings, proof-of-principle clinical studies are now warranted to test the therapeutic value of cutaneous ECS-targeted approaches in the clinical

management of multitude of human skin diseases. In the human sweat gland, it is envisaged that agents increasing the cutaneous endocannabinoid ‘‘tone’’ (such as employing endocannabinoids or molecules that upregulate the production of endocannabinoids by for example stimulating their synthesis or inhibiting their degradation) may be successfully applied in certain sweat gland disorders (e.g., benign or malignant tumors) characterized by unwanted cell growth. Likewise, modulators of the ECS and TRP channels, via influencing DC functions, may be effective tools in controlling the functions of the skin immune system. Actually, we have previously suggested similar approaches for the management of various growth and inflammatory conditions of the human pilosebaceous unit (e.g. hair growth problems, acne vulgaris). Finally, from industrial and social point-of-views, it is noteworthy that the proper control of excessive sweating e.g. by modulating the activity of the endocannabinoid-coupled signaling in the sweat gland epithelium, will surely attract the intense interest of the cosmetics industry.

Summary

In the present work, as part of our extensive research effort on cutaneous functions, we aimed at examining the endocannabinoid system (ECS) on human sweat gland cells and monocyte-derived dendritic cells (DCs).

Our results show that, on the human eccrine sweat gland-derived NCL-SG3 cell line, endocannabinoids dose-dependently suppressed proliferation, induced apoptosis, altered expressions of various cytoskeleton proteins (e.g., cytokeratins), and upregulated lipid synthesis. Interestingly, neither the metabotropic CB cannabinoid receptors, nor the "ionotropic" TRP ion channels, expressed by these cells, mediated the cellular actions of the endocannabinoids. However, the endocannabinoids selectively activated the mitogen-activated protein kinase signaling pathway. Finally, other elements of the ECS (i.e., enzymes involved in the synthesis and degradation of endocannabinoids) were also identified on NCL-SG3 cells.

On human monocyte-derived DCs, our goal was to evaluate the effect of heat shock on the endocytotic activity of the cells, and to elucidate the role of themosensitive transient receptor potential (TRP) channels in the process. We have shown that heat shock decreased the endocytotic activity of the DCs and that this effect could be alleviated by the RNAi-mediated knockdown of TRPV2. Likewise, the heat shock-induced robust membrane currents were selectively and markedly inhibited by TRPV2 "silencing".

Taken together, our pre-clinical data suggest that the targeted manipulation of the activity of the cutaneous ECS might be exploited in the future in the clinical management of a multitude of human skin conditions (including e.g. sweat gland-derived tumors, inflammation, excessive sweating, and other diseases of the human pilosebaceous unit and the related adnexal structures).

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List of publications related to the dissertation

1. **Szöllősi, A.G.**, Oláh, A., Tóth, I.B., Papp, F., Czifra, G., Panyi, G., Bíró, T.: Transient receptor potential vanilloid-2 mediates the effects of transient heat shock on endocytosis of human monocyte-derived dendritic cells.
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