SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (Ph.D.)

Role of the endocannabinoid system in the regulation of biological processes of human skin derived cells

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DOCTORAL SCHOOL OF MOLECULAR MEDICINE

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The Examination takes place at Departement of Biophysics, University of Debrecen
11 am, 29 April 2011.

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The Ph.D. Defense takes place at the Lecture Hall of the 1st Department of Medicine, Institute for Internal Medicine, Medical and Health Science Center, University of Debrecen
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INTRODUCTION

Research efforts of the last two decades have unambiguously confirmed that the human body is able to produce various molecules which exhibit biological effects similar to (-)-Δ^9-tetrahydrocannabinol, which can be found in the plant Cannabis sativa. These substances are the endogenous cannabinoids, among which the best known is the N-arachidonoyl ethanolamine (anandamide). Classically, CB1-mediated effects were mostly described in the central nervous system. Peripherally, the ECS has been implicated in the regulation of *e.g.*, immune and cardiovascular processes, chiefly *via* CB2-coupled signaling. Elements of the ECS were extensively documented in other tissues and organs, *e.g.* in the skin.

We have recently identified the functional existence of the cannabinoid receptors and endocannabinoids (AEA, 2-AG) in different cell types of the hair follicle. The other member of the pilosebaceous unit, the sebaceous gland cells reportedly also show CB receptor immunoreactivity *in situ*. However, direct evidence for the presence of a functional ECS in sebaceous glands and a description of the potential effects of endocannabinoids on various biological processes of human sebocytes are lacking. In the first part of the Ph.D. thesis, we have therefore analyzed the presence and function of the ECS and related signaling mechanisms in human sebaceous gland-derived cells, using the SZ95 sebocyte cell line, one of the best established human sebocyte cell culture models.

Cannabinoid receptors and enzymes in the metabolism of AEA were already idenified in normal human epidermal keratinocytes and immortalized HaCaT keratinocytes as well. However, none of the studies have investigated

the role of endocannabinoids in the regulation of human epidermal keratinocyte proliferation. Therefore, in the second part of the Ph.D. thesis, we have investigated the actions of the most extensively studied endocannabinoid, AEA, on the biology of human keratinocytes *in vitro* as well as *in situ*.

BACKGROUND

Cannabinoids

A group of compounds isolated from *Cannabis sativa*, their metabolites and synthetic analogues are called cannabinoids. This plant contains more than 420 different substances, most importantly (-)-Δ^9-tetrahydrocannabinol ((-)-Δ^9-THC), cannabidiol (CBD) and cannabinol (CBN). The prime psychoactive component, (-)-Δ^9 –tetrahydrocannabinol ((-)-Δ^9 –THC) also has analgetic and neuroprotective effects.

N-arachidonoylethanolamide or anandamide (AEA) is the first endogenous cannabinoid to be discovered, as such it has been intensively studied.. It is a derivative of arachidonic acid (AA), is composed of 22 carbon atoms and is a eicosanoid. *In vivo* it is formed from a membrane lipid precursor, *N-arachidonoil-phosphatidol-ethanol-amide* (NAPE). The 2-AG (*2-arachidonoyl glycerol)*, which also belongs to the eiocosanoid group, is produced from diacyl-glycerol (DAG) with the help of the DAG-lipase. The endocannabinoids are not stored, but formed and liberated according to the needs of the body. A selective uptake mechanism, the so called anandamide membrane transporter (AMT) eliminates the endocannabinoids from the

extracellular space shortly after their production. Subsequently they will be metabolised in the cells by fatty-acid-amide-hydrolase (FAAH) or monoacylglycerol-lipase (MAG-lipase) in case of 2-AG. The endovanilloid AEA, formerly thought to be an endocannabinoid, is capable of directly activating the transient receptor potential vanilloid 1 (TRPV1). Moreover, the activation of TRPV1 (with capsaicin for example) results in AEA release from sensory neurons. Focusing on the endocannabinoids raises the number of the newly discovered synthetic analogues, among which are the CB1 agonist *arachidonoyl-2-chloro-ethylamide* (ACEA) and CB2 agonist JWH-015, which we used during the experiments.

Cannabinoid receptors

The cannabinoid receptor type 1 (CB1), first described in the central nervous system, has a regulatory function in synaptic processes, motor learning and memory. This 60 kDa receptor is built up of 473 amino acids and has a 7-transmembrane (7-TM) protein structure. As a metabotropic receptor, the CB1 is involved in G-protein mediated signaling, where the inhibition of adenilate-cyclase (AC) leads to the decrease of cAMP, which will lower the phosphorilation of proteins by inhibiting protein kinase A (PKA). The concentration of cAMP depends on the type of G protein associated with the receptor, as it can also be agonistic. Ca^{2+} and K^{+} channels would open due to the activation of G proteins but the stimulation of the receptor might also launch the expression of certain genes via the mitogene activated protein kinase (MAPK) signaling pathway.

The cannabinoid receptor type 2 (CB2) is expressed mainly in immune and hemopoetic cells (spleen, thymus, tonsils, T and B cells). It

consists of 360 amino acids and shows a significant conformity with the CB1, their transmembrane regions are 68% homologous. Similarly to the CB1, the CB2 acts by inhibiting the AC and decreasing the intracellular cAMP levels. The receptor is able to stimulate the MAPK, and there is evidence that it causes changes in gene expression through the activation of the Erk cascade.

Cannabinoids in the skin

Both receptors were shown to be expressed in the human skin in significant amounts (epidermal keratinocytes, epithelial cells of the hair follicle, sebocytes and mast cells). It was also observed that both HaCaT and NHEK cells contain enzymes which play a role in the metabolism of AEA, and can synthetize endocannabinoids. At the same time it has been proven, that the stimulation of these receptors has an effect on other processes, like tumorigenesis, pain, itching or the growth of hair. Our laboratory recently confirmed the functional existence of CB1 in the regulation of the hair follicle; endo- and exocannabinoids reduce the elongation of the hair and the proliferation of matrix keratinocytes and induce enhanced apoptosis in human hair follicles.

The model of the sebaceous gland and the sebocytes: SZ95 cell line

The sebaceous gland is located in the dermis and with the hair follicle it forms the so called pilosebaceous unit. It has numerous paracrine, endocrine and immunological functions and assists in the normal homeostasis of the skin. Sebocytes secrete the sebum, which is rich in neutral lipids. The

altered capability of the sebocytes to proliferate or differentiate plays a central role in a vast number of skin diseases (rosacea, seborrhea and acne).

The *in vitro* model for human sebocytes is the SZ95 cell line, which bears the original phenotype of sebocytes, for example it stores a decent amount of lipids in the cytoplasm and also grows in size during differentiation. *In vitro* experiments with immortalized SZ95 cell lines revealed that the peroxisoma proliferator activated receptors (PPAR) located in the nucleus play an important role in the accumulation of lipids during the cell differentiation. It has also been demonstrated, that the differentiation of sebocytes can be induced by AA besides PPAR ligands, which leads to the aggregation of lipids and apoptosis of cells. AA and presumably PPAR are able to increase the production of proinflammatory citokines and other lipid-natured mediators in SZ95 cells as well.

The model of human epidermal keratinocytes: HaCaT keratinocytes

Keratinocytes are settled in the outermost layer of the skin, creating a barrier between the internal parts and the outer world and are responsible for defending against harmful environmental effects. To fulfill this task the keratinocytes proliferate and differentiate constantly, building up the characteristic layers of the epidermis. They are also capable of producing several important autocrine, paracrine and endocrine mediators. For instance they can synthetize many citokines and growth factors as a response to certain stimuli (UV radiation, tumor promoters) *in vivo* and *in vitro*. The released substances will activate particular cells (neutrophil granulocytes, memory T cells), this way the keratinocytes can be involved in the immune response against pathogens and antigens reaching the skin. In addition to this

the cytokines might activate definite pathways (eg. tyrosine kinases) by binding to specific receptors and creating complex signaling networks. Normal human epidermal keratinocytes (NHEK) die shortly in primary cultures thanks to triggered differentiation and apoptosis. For this reason it was necessary to create a cell line which is an apropriate *in vitro* model for NHEK processes and at the same time can be bred for an extensive period. The created cell line is called *H*uman *A*dult skin keratinocytes, low Ca^{2+}, elevated *T*emperature (or HaCaT).

Aims

In the current study, we intended to analyze the functional existence of the ECS and related signaling mechanisms in human sebaceous gland-derived SZ95 sebocytes (a human sebocyte cell culture model of sebaceous gland). Specifically, the following aims were defined:

- To describe the CB receptor pattern and the expression of certain endocannabinoids (AEA, 2-AG) in SZ95 sebocytes
- To evaluate the effects of endocannabinoids on various sebocyte functions (e.g. lipid synthesis, cell growth and death, gene expression) using numerous cellular and molecular assays
- To define the involvement of CB receptors and various intracellular signaling pathways in mediating the effects of endocannabinoids (by employing specific agonists, antagonists, and RNA interference)

However, none of the above studies has investigated the role of *endocannabinoids* in the regulation of human epidermal keratinocyte proliferation. Therefore, we have investigated the actions of the most

extensively studied endocannabinoid, i.e. AEA, on the biology of human keratinocytes. Our specific aims were:

- To define the *in vitro* and *in situ* effects of AEA on epidermal keratinocyte growth and survival;
- To identify those intracellular signaling pathways that may mediate the actions of the endocannabinoid.

To achieve these goals, we used human cultured keratinocytes (NHEK, HaCaT) and skin organ-culture models and employed combined pharmacological and molecular approaches.

MATERIALS AND METHODS

Cell culturing

Human immortalized SZ95 sebocytes, derived from human sebaceous gland, were cultured in Sebomed basal medium. NHEK were cultured in EpiLife serum free medium. Human immortalized HaCaT keratinocytes were cultured in Dulbecco's Modified Eagle's Medium. Cells were cultured at 5% CO_2 athmosphere, and 37 ºC.

Immuncytochemistry and immunhistochemistry

Cells were fixed in acetone and then permeabilized by 0.1% Triton-X in phosphate-buffered saline (PBS). After washing in PBS solution and blocking in 1% bovine serum albumin (BSA), cells were incubated overnight with primary antibodies at 4°C. For fluorescence microscopy, slides were incubated with a goat anti-rabbit fluorescein-isothiocyanate (FITC)-conjugated secondary antibody and nuclei were visualized using DAPI. As negative controls, the appropriate antibody was either omitted from the procedure or was pre-incubated with synthetic blocking peptides.

Normal skin samples, obtained during plastic surgery, were fixed in 4% buffered formalin (24 hrs), embedded in paraffin wax, and 4 μm thick sections were obtained. After antigen retrieval (8 mM Tris buffer pH 8.4 for 15 min in a microwave oven), endogenous peroxidase activity was blocked with peroxidase blocking solution. Non-specific binding was prevented by incubating the sections with Protein Block Serum Free Reagent. The tissue sections were then incubated overnight at 4 °C with the primary antibodies. Sections were then incubated with the EnVision+® System Labeled polymer-

HRP Anti-Rabbit with 3,3'-Diaminobenzidine (DAB) visualization. Tissue samples were finally counterstained with hematoxylin and mounted in aqueous mounting medium. As negative controls, the appropriate primary antibodies were pre-absorbed with synthetic blocking peptides.

To simultaneously assess proliferation and apoptosis in human skin organ cultures, a Ki-67/TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) double-staining method was employed. After AEA treatment, cryostat sections were first labeled with a digoxigenin-dUTP (ApopTag Fluorescein In Situ Apoptosis Detection Kit) in presence of terminal deoxynucleotidyl transferase (TdT), then with a mouse anti-Ki-67 antiserum. TUNEL+ cells were visualized by an anti-digoxigenin FITC-conjugated antibody (ApopTag kit) whereas Ki-67+ cells were labeled with a rhodamine-conjugated goat anti-mouse secondary antibody. Sections were then counterstained with DAPI. Negative controls were performed by omitting TdT and the Ki-67 antibody.

Western blotting

Cells were harvested in lysis buffer supplemented with a protease inhibitor cocktail and the protein content of samples was measured by a BCA protein assay. The samples were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gelelectrophoresis, transferred to BioBond nitrocellulose membranes and then probed with antibodies. HRP–polymer-conjugated, respective anti-rabbit or anti-mouse IgG antibodies were used as secondary antibodies and the immunoreactive bands were visualized by SuperSignal® West Pico Chemiluminescent Substrate enhanced chemiluminescence.

Immunoblots were the subjected to densitometric analysis using an Intelligent Dark Box and the Image Pro Plus 4.5.0 software.

RT-PCR

Total RNA was isolated using TRIzol according to the manufacturer's protocol, and the isolated total RNA was reverse-transcribed into cDNA and then amplified on a GeneAmp® PCR System 2400 DNA Thermal Cycler. PCR products were visualized on a 1,5 % agarose gel with ethidium bromide under UV and the photographed bands were quantified by an 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 using the 5' nuclease assay. PCR amplification was performed by using specific TaqMan primers and probes.

Determination of viable cell number

The number of viable cells was determined by measuring the conversion of the tetrazolium salt MTT to formazan by mitochondrial dehydrogenases. Cells were plated in 96-well plates in quadruplicates and were cultured for 24-48 hrs. Cells were then incubated with 0.5 mg/ml MTT at 37 °C for 3 hrs and the concentration of formazan crystals (as an indicator of the viable cell number) formed from tetrazolium salt in the mitochondria, was determined colorimetrically (A_{550}).

Determination of apoptosis

Apoptosis was determined by fluorimetric measurement of activation of pro-apoptotic caspases using a fluorescent inhibitor of caspases (Poly Caspases Detection Kit). The fluorescent FLICA reagent specifically and covalently interacts with the active centers of activated caspases via a caspase-specific recognition sequence. Keratinocytes were incubated with FLICA reagent and fluorescence was measured at 490 nm excitation and 530 nm emission wavelengths using FLIPR.

In addition, apoptosis was also assessed by measuring phosphatidyl-serine translocation with FITC-conjugated Annexin-V. Fluorescence was measured at 490 nm excitation and 520 nm emission wavelengths using FLIPR.

The abolishment of mitochondrial membrane potential is one of the earliest markers of apoptosis. To assess the process, mitochondrial membrane potential of cells was determined using a MitoProbe™ $DiIC_1(5)$ Assay Kit. Cells (15,000 cells/well) were cultured in 96-well black-well/clear-bottom plates in quadruplicates and were treated with various compounds for 24-48 hrs. After removal of supernatants, cells were incubated for 30 minutes with $DilC_1(5)$ working solution (30 µl/well), then washed with PBS, and the fluorescence of $DilC_1(5)$ was measured at 630 nm excitation and 670 nm emission wavelengths using the above FLIPR. RFU values were expressed as percentage of vehicle controls regarded as 100 %.

In addition, another hallmark of apoptosis (i.e. membrane perturbation) was also assessed by flow cytometry. Following treatment with various agents, cells were harvested and stained with an Annexin-V-FITC/Propidium Iodide Apoptosis Kit following the manufacturer's protocol. Fluorescence intensity was measured by a Coulter Epics XL flow cytometer.

Determination of necrosis/cytotoxicity

Necrotic cell death was first determined by measuring glucose-6-phophate-dehydrogenase (G6PD) release. The enzyme activity was detected by a two-step enzymatic process that leads to the reduction of resazurin into red-fluorescent resorufin. Cells (15,000 cells/well) were cultured in 96-well black-well/clear-bottom plates in quadruplicates and treated with various compounds for 24-48 hrs. A 2x reaction medium was then prepared according to the manufacturer's protocol, added to the wells in 1:1 dilution, and incubated at 37 ^{o}C for 15 minutes. The fluorescence emission of resorufin was monitored by the FLIPR device at 545 excitation and 590 emission wavelengths.

The cytotoxic effects of cannabinoid treament was also determined by SYTOX Green staining. The dye is able to penetrate only to necrotic cells with ruptured plasma membranes. Cells were cultured in 96-well black-well/clear-bottom plates and were treated with various compounds for 24-48 hrs. Supernatants were then discarded and the cells were incubated for 30 minutes with 1 μM SYTOX Green solution. Fluorescence of SYTOX Green was measured at 490 nm excitation and 520 nm emission wavelengths using FLIPR.

Determination of intracellular lipids

For semi-quantitative detection of sebaceous lipids, cells were cultured on glass coverslips and treated with several compounds for 24-48 hrs. Cells were fixed in 4% paraformaldehide for 1 h, washed in PBS and once in 60% isopropanol, stained in freshly prepared Oil Red O solution (in

60% isopropanol). Nuclei were counterstained with Mayer's hematoxylin. Stained cells were mounted in aquaeus mounting medium.

For quantitative measurement, cells (15,000 cells/well) were cultured in 96-well black-well/clear-bottom plates in quadruplicates and were treated with compounds for 24-48 hrs. 100 µl of a 1 µg/ml Nile red solution in PBS was added to each well. The plates were then incubated at 37°C for 20 min, and the emitted fluorescence was measured on FLIPR. Neutral lipids were measured at 485 nm excitation and 565 nm emission wavelengths, while 540 nm excitation and 620 nm emission wavelengths were used for the detection of polar lipids.

RNA interference (RNAi)

Cells at 50-70 % confluence were transfected with specific Stealth RNAi oligonucleotides against CB_1, CB_2, TRPV1 using Lipofectamine 2000 according to the manufacturer's protocol. For controls, RNAi Negative Control Duplexes (Scrambled RNAi) were employed. The efficacy of RNAi-driven "knock-down" was daily evaluated by Q-PCR and Western blotting for 4 days.

Calcium measurement

Keratinocytes, cultured on glass coverslips, were loaded with a calcium sensitive probe 5 µM fura-2 AM and were then placed on the stage of an inverted fluorescence microscope. Excitation was alternated between 340 and 380 nm using a dual wavelength monochromator. The fluorescence ratio (F_{340}/F_{380}) values were determined.

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.

Data were analyzed using One-Way ANOVA with Bonferroni and Dunnett post-hoc probes, and $p<0.05$ values were regarded as significant differences.

RESULTS

I. The functional roles of the endocannabinoid system in human sebocytes

CB expression in human sebaceous gland and human SZ95 sebocytes

First, we intended to identify the existence of CB receptors on the human sebaceous gland *in situ* and on human SZ95 sebocytes. Specific *in situ* CB1 and CB2 immunoreactivity was identified on epithelial cells of the sebaceous gland. However, using immunocytochemistry, Western blotting and RT-PCR on SZ95 sebocytes, we were able to identify only the relatively high expression of CB2 whereas the appearance of CB1, was around the detection limit.

Human SZ95 sebocytes produce endocannabinoids

We also tested whether SZ95 sebocytes synthesize endocannabinoids. Using mass spectrometry, we were able to show that SZ95 sebocytes express both AEA (66,7±10 fmol/mg tissue) and 2-AG (6,2±1 pmol/mg tissue) (means±se, n=4) at levels similar to those detected earlier in

various skin samples; *e.g.*, 50 fmol/mg tissue AEA in rat paw and mouse ear samples or 20–30 pmol/mg tissue 2-AG in mouse ear skin.

Endocannabinoids enhance lipid synthesis and induce apoptosis in SZ95 sebocytes

As revealed by Oil Red-O staining and quantitative Nile Red-based fluorimetry, both AEA and 2-AG markedly and dose-dependently enhanced neutral lipid accumulation in the cells, reflecting stimulation of sebocyte differentiation. Endocannabinoids were also shown to decrease cellular viability (MTT assay). To assess whether this latter effect was due to apoptosis and/or necrosis, first flow cytometry analysis was performed. Both AEA and 2-AG markedly increased the number of Annexin-V-positive cells whereas they only slightly elevated the number of double Annexin-V- and PI-positive sebocytes. In a series of quantitative fluorimetric assays, AEA was found to markedly decrease mitochondrial membrane potential (another hallmark of apoptosis) in a dose-dependent fashion whilst only the highest concentration of AEA was able to moderately increase SYTOX Green accumulation and G6PD release, two complementary indicators of necrosis/cytotoxicity.

The cellular effects of AEA are mediated by CB2

Our laboratory has recently described the functional existence of TRPV1 in SZ95 sebocytes; we also measured the effect of the TRPV1 antagonist I-RTX on the cellular action of AEA. As seen in inhibition of TRPV1 had no effect on the action of AEA to enhance lipid synthesis. We then investigated whether the cellular mechanism of action of AEA on SZ95 sebocytes was mediated by the existing CB receptors. The synthetic CB2-

specific agonist JWH-015 – but, not the CB1-specific agonist ACEA – mimicked the action of the endocannabinoids to enhance lipid synthesis and to induce cell death. We also found that the cellular effects of AEA were significantly prevented by the CB2 antagonist AM-630 whereas the CB1 inhibitor AM-251 was ineffective.

To further assess the role of CB2, a series of RNAi experiments against the receptors were carried out. Western blot and RT-PCR analysis revealed that the expression of CB2 was significantly "knocked-down" by all 3 RNAi probes at day 2 after transfection and remained suppressed also on day 3. We then investigated the effects of AEA-treatment (24 hrs) on the lipid synthesis of RNAi-transfected cells on day 2. AEA was unable to enhance lipid synthesis at all (CB1-targeted RNAi did not affect the cellular action of the endocannabinoid). Intriguingly, in sebocytes with RNAi-mediated "knock-down" of CB2, we found significantly decreased basal lipid content as well which data argue for the endogenous role of CB2-mediated signaling in the regulation of lipid synthesis of the cells.

The CB2-mediated cellular signaling involves the MAPK pathway

We intended to define components of the CB2-mediated cellular signaling in human sebocytes. The effect of AEA to enhance lipid synthesis was not modified by the inhibition of the PKC (by GF109203X) or the PI3K (by Wortmannin) systems. In contrast, the MAPK inhibitor PD098059 significantly prevented the action of AEA, to similar extent to the effect of the CB2 antagonist AM-630. The involvement of the MAPK pathway in the endocannabinoid-induced lipid synthesis was also suggested by the fact that

both AEA and 2-AG induced a marked, transient phosphorylation of the MAPK Erk-1/2, reflecting activation of the signaling pathway.

Endocannabinoids up-regulate expression of genes involved in the regulation of lipid synthesis

We investigated the effect of endocannabinoid treatment on the expression of PPAR isoforms in SZ95 sebocytes. As assessed by Q-PCR analysis, both AEA and 2-AG significantly up-regulated expression of PPARδ and γ whereas we found significantly elevated PPARα gene levels only in the 2-AG-stimulated group treated for 24 hrs. We also tested whether endocannabinoid treatment affects the expression of PPARγ target genes– COX-2 (cyclooxygenase-2), ADRP (adipose differentiation-related protein), PGAR (PPARγ-regulated angiopoietin-related protein). Both AEA and 2-AG dramatically elevated the expression levels of all target genes investigated, further supporting the activation of PPARγ. This latter finding was also strengthened by the fact that the effect of AEA to stimulate lipid accumulation in SZ95 sebocytes was significantly prevented by GW9662, a selective inhibitor of PPARγ.

II. ***The effect of AEA in human epidermal keratinocytes-roles of the transient receptor potential vanilloid-1 and the cannabinoid receptors***

Cultured human keratinocytes express CB1, CB2, and TRPV1

Expressions of CB1 and CB2 were identified both on NHEK and HaCaT keratinocytes, using complementary immunocytochemistry and Western blotting techniques. Likewise, TRPV1 was also detected on these

cells. This corresponded well to expression of the CB1 and CB2 genes in both types of keratinocytes, as demonstrated by RT-PCR and by quantitative "real-time" PCR.

AEA inhibits human keratinocyte growth and induces cell death

We found that AEA dose-dependently reduced cell viability and proliferation of both NHEK and HaCaT cells. As measured by quantitative fluorimetric determinations, AEA markedly and dose-dependently increased the number of Annexin-V-positive cells. Moreover, the endocannabinoid AEA markedly decreased mitochondrial membrane potential and induced the activation of caspases. The highest concentrations of AEA was also able to significantly increase SYTOX Green accumulation and G6PD release, two complementary indicators of necrosis/cytotoxicity.

AEA inhibits proliferation and induces apoptosis of epidermal keratinocytes in situ

We employed the organ culture of normal, full-thickness human skin fragments. After treatment with AEA, double Ki67/TUNEL immunolabeling was performed to simultaneously assess the *in situ* effects of AEA on human keratinocyte proliferation and apoptosis. AEA treatment markedly suppressed the percentage of proliferating (Ki67+) whereas it dramatically increased that of apoptotic (TUNEL+) cells in normal human epidermis organ-cultured under serum-free conditions.

Cellular actions of AEA are mediated by CB1 and TRPV1

Cultured human keratinocytes were treated with highly selective inhibitors of defined CB subtypes (AM251 for CB1, AM630 for CB2) or

TRPV1 (CPZ, I-RTX). These inhibitors did not reduce viability of the cells. However, inhibition of CB1 and TRPV1, but not of CB2, markedly abrogated the growth-inhibitory and apoptosis-inducing cellular effects of AEA. The suppression of extracellular [Ca^{2+}] also prevented the cellular actions of AEA, further supporting the involvement of the Ca^{2+}-permeable ion channel TRPV1.

To further assess the roles of CB1 and TRPV1, a series of receptor knock-down experiments were carried out. Scrambled RNAi probes or RNAi oligonucleotides against CB1, CB2, and TRPV1 did not decrease human keratinocyte viability in culture. In fact, RNAi-mediated silencing of CB1 and TRPV1 stimulated keratinocyte growth. This latter finding suggested that CB1 and TRPV1 may function as constitutively active and/or continuously activated receptors to inhibit the growth of epidermal keratinocytes. Similar to the pharmacological data, silencing of CB1 or TRPV1 counteracted AEA's negative effect on cell viability and the induction of apoptosis.

A sequential signaling pathway (CB1 → TRPV1 → Ca^{2+}-influx) mediates the actions of AEA on human keratinocytes

To determine the possible interactions between CB1 and TRPV1, fluorescent calcium measurements were performed on a FLIPR. Keratinocytes were loaded with fura-2 AM. As a positive control, we employed the TRPV1 agonist capsaicin (CPS) which increases $[Ca^{2+}]_i$ in keratinocytes. AEA induced transient elevations of $[Ca^{2+}]_i$ in a dose-dependent manner. Amplitude of the maximal AEA-induced $[Ca^{2+}]_i$ elevation was in the range of that evoked by 10 μM CPS. However, the kinetics of the AEA- and the CPS-induced cellular actions were markedly different.

Namely, the effect of AEA was realized only after a long incubation of the cells (time-to-peak, TTP), in contrast to the fast action of the TRPV1 agonist CPS. The AEA-evoked $[Ca^{2+}]_i$ transients were completely prevented by administering the TRPV1 antagonists CPZ or I-RTX, or by suppression of extracellular $[Ca^{2+}]$. Likewise, the effect of AEA to raise $[Ca^{2+}]_i$ was also fully abrogated by the CB1 antagonist AM-251. Finally, we have also found that co-administration of CB1 and TRPV1 antagonists did not exert additive inhibitory effects. In contrast, the selective CB1 antagonist AM-251 markedly further increased TTP value almost threefold. The CB2 antagonist AM-630 had no effect on either the amplitude or the TTP of the AEA-evoked $[Ca^{2+}]_i$ elevations. We also found that the effect of CPS to raise $[Ca^{2+}]_i$ was markedly abrogated by the TRPV1 inhibitors as well as by suppression of extracellular $[Ca^{2+}]$, whereas it was not affected by antagonists of CB_1 or CB_2.

DISCUSSION

Human SZ95 sebocytes possess a functional, constitutively actice endocannabinoid system

Since previous reports have extensively documented that endocannabinoids might exert their cellular actions via CB1, CB2, and/or (especially in the case of AEA) via TRPV1-coupled signaling mechanisms and, furthermore, since we have recently described the functional existence of TRPV1 both in the human sebaceous gland and in SZ95 sebocytes; a central part of our study was to identify the molecular target(s) of the endocannabinoids. Our results that (i) the cellular effects of endocannabinoids were abrogated by the CB2-specific antagonist AM-630 but not by TRPV1 or CB1 antagonists; (ii) similarly, endocannabinoids were ineffective on those sebocytes in which CB2 expression were selectively "knocked-down" by RNAi; (iii) the effects of endocannabinoids were fully mimicked by the synthetic CB2-specific agonist JWH-015 but not by the CB1-specific agonist ACEA; (iv) CB2 was successfully identified in the cells (both at the protein and mRNA levels) whereas the expression of CB1 was uncertain; suggest that endocannabinoids may exclusively act on CB2 to enhance lipid synthesis and induce cell death of human sebocytes.

We also intended to define the "down-stream" cellular target mechanisms following CB2 activation. Among the multiple intracellular signal transduction systems (e.g. PKC, MAPK, PI3K), which were previously shown to be modulated by cannabinoids in certain cells types, we found the MAPK pathway to mediate the CB2-induced cellular actions of

endocannabinoids in human sebocytes. We moreover provide the first evidence that the activation of endocannabinoid-CB2-MAPK signaling also results in induction of certain nuclear receptors (recognized regulators in lipid homeostasis in various cell types), suggesting that, when activated, the ECS may significantly modulate the global gene expression profile of the cells.

It was also intriguing to observe that the overall cellular modifications (i.e. enhancement of lipid synthesis, induction of apoptosis) induced by endocannabinoids strikingly resemble those seen in acne vulgaris, a common, multi-factorial pilosebaceous inflammatory skin disease in which differentiation and hence lipid synthesis of sebocytes are pathologically increased. Collectively, these findings raise a novel question whether *pathologically augmented* ECS-CB2 signaling contributes to acne pathogenesis. Although further studies are invited to systemically explore how the endocannabinoid signaling can be manipulated in a clinically desired manner in the treatment of acne (and possibly other relevant sebaceous gland diseases), our novel presentation that, in "CB2-silenced" SZ95 sebocytes, not only the AEA-stimulated but also the *basal* lipid synthesis was significantly suppressed can be interpreted as a first, tentative proof-of-principle for a novel, CB2-targeted treatment option in the clinical management of acne.

A comparison of available data in the literature with our current and recent findings highlights other important, both physiologically and clinically relevant aspects of the ECS in human and murine skin biology. Namely, cannabinoids via CB1-mediated signaling were shown to inhibit human hair growth and induce apoptosis-driven regression in the hair follicle as well as to suppress differentiation of epidermal keratinocytes. However, both CB1 and CB2-coupled mechanisms were suggested in suppressing murine and

human skin tumor growth as well as attenuating allergic contact dermatitis in mice. Along these lines, our current presentation that the *endogenously active* ECS enhances lipid synthesis and induces cell death selectively via CB2 strongly argues for the existence of cell type-specific and (most probably) receptor-selective regulatory endocannabinoid mechanisms in the skin.

Effects of AEA are mediated by a sequentially engaged signaling mechanism of CB1 and TRPV1

We provide the first evidence that the prototypic endocannabinoid AEA –which is synthesized in several human skin cell compartments – inhibits proliferation and induces cell death of human epidermal keratinocytes in cultures as well as *in situ*. We also show that AEA-induced keratinocyte death is Ca^{2+}-dependent. These data support the concept that keratinocytes exploit a physiologically relevant ECS for negatively regulating their own growth in a paracrine and/or autocrine manner.

Furthermore, we show that the keratinocyte death-promoting effects of AEA are mediated by a sequentially engaged signaling mechanism (CB_1 $\rightarrow$ TRPV1 $\rightarrow$ Ca^{2+}-influx). This model is supported by several lines of evidence:

(i) Both CB_1 and TRPV1 antagonists, the suppression of extracellular [Ca^{2+}], and RNAi-mediated silencing of these receptors were able to prevent the cellular actions of AEA.

(ii) However, these pharmacological and molecular inhibitory effects were not additive, arguing for the lack of co-activation of CB_1 and TRPV1 by AEA.

(iii) The effect of AEA to increase $[Ca^{2+}]_i$ was realized only after a long incubation of the cells, in contrast to the immediate action of the "direct" TRPV1 agonist CAPS. Although we cannot exclude the possibility that AEA exhibited a slower onset of action due to its higher lipophilicity as compared to that of capsaicin, these results suggest that AEA may not directly activate TRPV1 but rather multiple (and yet to be determined) AEA-evoked signaling pathways are involved in the opening of the ion channel.

(iv) That these "upstream" mechanisms involve the preceding action of AEA on CB_1 is supported by the fact that whereas both CB1 and TRPV1 antagonists were able to equally suppress the amplitude of the AEA-induced $[Ca^{2+}]_i$ elevations, the CB_1 antagonist AM-251 (unlike the "channel antagonists" of TRPV1 which do not affect the activity of CB_1) markedly increased, the already very long, TTP value of the $[Ca^{2+}]_i$ transients.

(v) Finally, we found that the effect of capsaicin to raise $[Ca^{2+}]_i$ was inhibited by TRPV1 antagonists and by suppression of extracellular $[Ca^{2+}]$ but not by antagonists of CB_1 which argues for the lack of an "other way around" sequential mechanism of AEA $\rightarrow$ TRPV1 $\rightarrow$ CB1 $\rightarrow$ Ca^{2+}-influx.

Since inhibition or RNAi-mediated silencing of CB2 did not affect the cellular actions of AEA (which, otherwise, may also activate CB2), it appears that CB2 is not involved in mediating the growth-inhibitory effect of AEA on human epidermal keratinocytes. These results were in line with previous findings showing that CB1, but not CB2, plays a pivotal role in regulation of epidermal differentiation of human keratinocytes. However, CB2-mediated signaling on keratinocytes was shown to be involved in anti-nociception (by the release of endogenous opioids which, in turn, inhibit

pain-sensing skin afferent fibers) and in various forms of cutaneous inflammation.

That RNAi-mediated silencing of CB1 and TRPV1 significantly promoted the growth of human keratinocytes suggests that the joint CB1-TRPV1 signaling pathway identified here functions as a previously unknown, endogenously active receptor-channel mechanism that constitutively keeps human keratinocyte proliferation in check. Therefore, the fine-tuned endogenous ECS tone of the skin – set by constant or on-demand production of locally synthesized endocannabinoids – not only controls e.g. cutaneous immune competence and/or tolerance, lipid homeostasis, or adnexal biology, but also regulates epidermal homeostasis. Obviously, subsequent studies will need to support the physiological relevance of this novel concept by direct *in vivo* evidence. Likewise, it deserves systematic analysis whether dysfunctions of the cutaneous ECS can trigger or aggravate chronic hyperproliferative, pruritic, and/or pro-inflammatory skin diseases. Along these lines, the data reported here certainly encourage one to explore whether the targeted manipulation of the ECS could become a useful adjunct treatment strategy for hyperproliferative human dermatoses such as e.g. psoriasis or keratinocyte-derived skin tumors.

SUMMARY

We have investigated the role of the endocannabinoid system in the regulation of key biological processes of human epidermal keratinocytes and sebaceous gland-derived sebocytes. First we have identified the existence of the CB2 receptor in the sebaceous gland and on immortalized SZ95 sebocytes (the expression of CB1 was not confirmed). The endocannabinoid AEA and 2-AG, which were identified in SZ95 sebocytes, dose-dependently induced lipid production and (chiefly apoptosis-driven) cell death, and up-regulated the expression of genes involved in lipid synthesis (e.g. PPAR transcription factors and their certain target genes). These actions were selectively mediated by CB2-coupled signaling also involving the MAPK pathway. Since cells with "silenced" CB2 exhibited suppressed basal lipid production as well, our results suggest that human sebocytes utilize a paracrine-autocrine, endogenously active, CB2-mediated endocannabinoid signaling system for positively regulating lipid production and cell survival.

Using human cultured keratinocytes and skin organ-culture models, we have provided the first evidence that AEA markedly suppresses keratinocyte proliferation and induces cell death, most probably due to a Ca^{2+}-influx, both *in vitro* and *in situ*. Moreover, we also present that these cellular actions are mediated by a novel, most probably constitutively active signaling mechanism which involves the activation of the CB1 and a sequential engagement of the TRPV1. Hence, the data reported here may encourage one to explore whether the targeted manipulation of the above signaling pathway of the cutaneous ECS could become a useful adjunct treatment strategy for hyperproliferative skin diseases.

LIST OF PUBLICATIONS

Publications providing the basis of the thesis:

Dobrosi N, Tóth BI, Nagy G, Dózsa A, Géczy T, Nagy L, Zouboulis CC, Paus R, Kovács L, Bíró T. (2008): Endocannabinoids enhance lipid synthesis and apoptosis of human sebocytes via cannabinoid receptor-2-mediated signaling. *FASEB J. 22(10),* 3685-3695.**IF:7,049**

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Cumulative IF: 23,592