Role of the signalling mechanisms in the regulation of the biological processes of the skin

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INTRODUCTION

Functional anatomy of the skin; the HaCaT keratinocytes

Until the middle of the last century scientists found the skin to be only a barrier against mechanical, chemical and thermal stimuli. Recently it has been discovered that the skin has more widespread function, and this organ is under intensive investigation.

The **skin (cutis)** can be devided into three parts: *epidermis*, *dermis* and *tela subcutanea*. They can be further devided into anatomically and developmentally different parts.

The border between the outer environment and the body is the *epithelium* (epidermis). The following types of cells can be found here: keratinocytes, Merkel-cells, melanocytes, Langerhans-cells, and lymphocytes*.*

The biological functions of keratinocytes can be well examined in the HaCaT (*H*uman *A*dult skin keratinocytes, low *Ca*$^{2+}$, elevated *T*emperature) cell line. The cells originate from a man suffering from malignant melanoma. The keratinocytes were removed from that part of his skin which had not been transformed malignantly. The HaCaT keratinocytes shows genetic differences compared to the healthy skin, but they differentiate simliar to them.

Next part of the skin is the *dermis* (dermis). The following types of cells can be found here: *fibroblasts, histiocytes/macrophages, mast cells, adipocytes, undifferentiated mesenchymal cells, lymphocytes, plazmacells, eosinophil, basophil, neutrophil granulocytes*, and *monocytes*.

The inner part of the skin is the *hypodermis (*tela subcutanea).

Functional anatomy of the hair follicle

Hair follicle can be compared to an onion, where the dermal papilla (DP) represents the inner leaves of the onion, and DP is covered by the inner root sheath (IRS) and the outer rooth sheath (ORS). The onion's stem represents the growing hairshaft, which is produced by the keratinocytes, whereas the colour of the hair is determined by the melanin content of the melanocytes. The hairshaft grows together with the IRS's terminally differentiated keratinocytes (Henle, Huxley, cuticule), which covers the hairshaft. The slowly proliferating ORS composes the hairshaft's next layer and provides it with oxygen, nutrients and mediators modulating the hair growth.

The hair cycle

The follicle is characterized by a life-long regression and regeneration, called hair cycle. It has three different phases: the proliferating <u>anagen</u> phase, where the follicle is developed. Next phase is the regression or <u>catagen</u>, the elongation of the follicle stops here. Apoptosis takes place mainly in the layers of keratinocytes, whereas the decrease in the number of DP cells is the result of the cells' migration from here. The melanin content of the melanocytes diminishes during catagen because of the apoptosis. The following is the resting <u>telogen</u> phase, where the follicle is preparing for the next cycle. The molecules (cytokines, hormones and growth factors), that are able to influence the cycle are called positive (induce growing and proliferation) or negative (induce regression and apoptosis) regulators of the hair cycle.

The vanilloid (capsaicin) receptor-1 (TRPV1)

Capsaicin receptor is a non-specific cation channel (mostly permeable to cations), which is one of the most important integrator molecule of the responses evoked by painful stimuli.

The receptor is cloned and characterized by Caterina. The rat TRPV1 consists of 838 amino acids, weighing 95 kDa, and encoded by 2514 nucleotids. According to its structural features it shows homology with the transient receptor protein (TRP) located in the *Drosophila melanogaster's* retina , that's why it is the member of the TRP family. These receptors have six transmembrane domains, intracellular N-and C- terminals and can be found in tetramer forms.

The first molecule that can be related to the TRPV1 is the capsaicin (Capsaicum annuum) isolated from the hot pepper. The cellular effect of the capsaicin in the sensory neurons can be characterized by three processes: excitation, desensitization and neurotoxicity. The TRPV1 can be activated not only by exogen vanillod compounds, but also by the so called "endovanilloids".

Expression of the TRPV1

The TRPV1 was first written in a special group of the primary sensory neurons. Later it was described in different parts of the brain (cerebellum, cortex, striatum, bulbus olfactorius, pons, hippocampus and thalamus). The functional presence of the receptor is not restricted only to the neuronal tissue, because Bíró showed capsaicin-like effects also in mast cells and glioma cells. The capsaicin displays its effect also in human polymorphonuclear cells, lymphocytes, thymocytes, bronchial epithelium and in the epithelial, interstitial and smooth muscle cells of the urinary bladder.

Functional role of the TRPV1 in the follicle- previous results

We documented the expression pattern of TRPV1 in different compartment (epidermis) and cell types (Langerhans-cells, sebocytes, smooth muscle cells of the blood vessels, epithelial cells of the sweat gland, and mast cells) of the skin, whereas the melanocytes and fibroblasts were TRPV1 negative. In human hair follicle TRPV1-immunoreactivity (TRPV1-ir) could be seen in the layer of ORS, IRS and matrix keratinocytes, while the DP and melanocytes lacked the presence of the receptor. The TRPV1 –signal increased during catagen phase. Application of capsaicin dose-dependently decreased the elongation of the hairshaft, which effect could be prevented by iodo-resiniferatoxin (I-RTX), the specific antagonist of TRPV1. We also provided evidence that capsaicin decreased the number of proliferating, Ki67 positive cells and increased the ratio of TUNEL positive apoptotic cells. Stimulation of TRPV1 induced catagen transformation in most of the follicles.

HaCaT and ORS keratinocytes also express TRPV1. Capsaicin increased the $[Ca^{2+}]_i$, which could be prevented by I-RTX showing the functional presence of the receptor in these cells. There was no detectable change in the $[Ca^{2+}]_i$ after removal of the extracellular Ca^{2+}. Following capsaicin application flow-cytometric analysis showed increase in the level of Ki67 and the positive regulators of the hair cycle, while the level of annexin-V and negative regulators were decreased in the follicle, further arguing our results.

Cannabinoids

The cannabinoids derived their name from Δ9-tetrahydrocannabinol (THC), isolated from the leaf of *Cannabis sativa*. The cannabinoid compounds can be devided into three subgroups: *plant*, *endogen* and *synthetic cannabinoids*. $Δ^9$ –THC and $Δ^8$ –THC

belong to the group of plant cannabinoids. The first investigated endogen cannabinoid, *N-arachidonoylethanolamine* (anandamide, AEA) is the most often used eicosanoid molecule, contains 22 carbon atoms. As a retrograde messenger it inhibits the release of NMDA, Ach and 5-HT from the presynaptic membrane. *2-arachidonoylglycerol* (2-AG) is an eicosaniod, too. Analogues of the Δ^9 –THC are called synthetic cannabinoid.

Cannabinoid receptors

The gene of the cannabinoid receptor-1 (CB1) (first detected in 1990) is located in the 6th cromosome, in the q14-15 region. It consits of 473 amino acids, weighing 60 kDa and contains 7-transmembrane domains. CB1 is a metabotropic receptor, related to a G-protein. After activation of G-proteins, K^+ and Ca^{2+} channels can open, resulting the changes of the membrane potential. Stimulation of the receptor can also activate MAPK and so induces changes in the gene expression.

The mRNA of cannabinoid receptor-2 (CB2) was found in immune tissues first. The gene is located in the q36 region of the 1st chromosome. The protein consists of 360 amino acids and shows 44% homology with the CB1. In the transmembrane regions of the CB1 and CB2 there is 68% homology, which means a big similarity between the two receptors. The CB2 is (like CB1) also able to activate a G-protein through a 7-TM protein, can stimulate a MAPK and Erk cascade, the latter can cause changes in the gene expression pattern, respectively.

Cannabinoid receptors in the body

The CB1 was first discovered in the nervous system, where it was thought to regulate memory functions. Stimulation of the receptor can

be a useful treatment in demyelination disorders, epilepsy and hypeproliferative states.

CB2 is present mainly in the immune system. A smaller amount of CB2 was detected in the thymus, bone marrow, heart, lung, prostate, uterus, cerebellum and in the hippocampus.

The cannabinoid system in the skin

Growing number of papers explain about the expression of CB1 and CB2 in the different part and cell types of the skin. Both the HaCaT and the NHEK keratinocytes contain the enzymes participated in the metabolism of AEA. Activation of the receptors has anti-tumoral effects, it decreases the acute, neuropathic pain and inflammation without having CNS side-effects. The cannabinoids have antipruriceptive effects, too.

Effect of mechanical stimuli on the skin; mechanosensitive channels

Many types of cells are able to detect mechanical sitmuli in the skin. After a long exposition, the keratinocytes undergo hyperproliferation, that can lead to different skin diseases (hyperkeratosis).

Applying a mechanical stress on the surface of the skin, the mechanosensitive channels (MSC) open and both the membrane potential and the $[Ca^{2+}]_i$ changes within the cell. Increase of $[Ca^{2+}]_i$ can activate the PKC enzymes, which influences the proliferation and differentiation of the keratinocytes.

One part of the MSCs' regulate the cell volume. To change the cell volume one can increase or decrease the tonicity around the cell, activating or inactivating the MSCs and inducing the activation of second messenger pathways.

AIMS

1. We have reported in our previous work the functional role of TRPV1 in the biological processes of the human hair follicle. In our present experiments we wanted to investigate the similarity between the expression of TRPV1 in human and mouse skin. We compared the changes in the hair cycle between the two species and by the investigation of the TRPV1 knockout mice we analysed the changes of the hair cycle in the lack of TRPV1.

2. In the next part of our experiments we examined both the expression pattern of the CB receptors in the follicle and the differences in the hair cycle. We also investigated the effect of the cannabinoid molecules on the biological functions of the follicles. We analysed the effect of the AEA, 2-AG and Δ^9-THC on the elongation of the hairshaft, on the apoptotic and proliferative processes and on the time course of the hair cycle. Finally we studied the possible connection between the TRPV1 and CB in the regulation of human hairshaft elongation.

3. In the last part of our work we investigated the role of the mechanosensitive channels (MSC) in HaCaT keratinocytes. After applying different hypotonic stimuli we detected the changes in membrane potential and examined what kind of channels can be opened during this event. Finally we studied the role of MSCs in the regulation of the cell proliferation and differentiation.

MATERIALS AND METHODS

Isolation and maintenance of human hair follicles; culturing HaCaT keratinocytes

Human anagen HF (n=18-24 per group) were isolated from skin obtained from females undergoing face-lift surgery. Isolated HF were maintaned for 7-9 days in supplemented Williams E medium. Length measurements were daily performed on individual HF. Cryostat sections (8 μm thick) of cultured HF were embedded and fixed. We used the same samples for representing the whole skin.

We cultured the HaCaT keratinocytes in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% FBS and antibiotics.

TRPV1 knockout mice

A tissue bank was prepared from adolescent back skin of female C57BL/6 mice in which hair follicle cycling had been induced by depilation. This was used for immunohistological detection of hair cycle-associated differences in TRPV1 expression. The functional role of TRPV1 signaling was addressed by spontaneous, experimentally unmanipulated hair follicle cycling during the first murine hair cycle (P19–P45), comparing TRPV1 knockout B6.129S4-Trpv1 mice (Jackson Laboratory, Bar Harbor, MA) and their age-matched littermates. Cryostat sections of back skin (at least three animals each per time point) were processed for histology and counterstained by haematoxylin– eosin.

Histological and morphological examinations

By the investigation of the morphological properties of the human follicles, we performed haematoxylin- eosin staining on 8 μm thick,

aceton- fixed slides. To determine the exact melanin content of the follicles, we performed Masson-Fontana staining.

To compare the differences between the hair cycle stages of control and TRPV1 knockout mice, first we assigned the follicles into their respective stages according to the morphological criterias of anagen, catagen or telogen phase. Concerning to that criterias, there are 6 phases in anagen, 8 phases in catagen and only 1 phase in telogen. To specify the classification one can calculate the cumulative hair cycle score (qualitativ histomorphometry). Indeed, we assigned a score to each hair cycle phase (e.g. anagen I= 1 score) and multiplied it by a factor refers to that stage. Finally we got a value between 1 and 6 in case of anagen and 1-8 in catagen phase.

Immunocyto- and histochemistry

For the detection of TRPV1, CB1 and CB2 immunoreactivity, the tyramide-amplification (TSA) and a peroxidase- based ABC technique were performed. Sections were first incubated with a primary anti-TRPV1, anti-CB1 and anti-CB2 antibody with biotinylated multilink swine anti-goat/mouse/rabbit IgG, and then by a streptavidin–horseradish peroxidase. Finally, we applied fluorescein isothiocyanatetyramide or diamino-benzidine, respectively and then sections were counterstained.

Double immunolabeling of proliferating and apoptotic cells

To evaluate apoptotic cells in colocalization with a proliferation marker Ki-67, a Ki-67/TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) double-staining method was employed. Cryostat sections were fixed in formalin/ethanol/acetic acid and labeled with a digoxigenin-deoxyUTP in presence of terminal deoxynucleotidyl transferase (TdT), followed by incubation with a mouse anti-Ki-67

antiserum. TUNEL+ cells were visualized by an antidigoxigenin FITC-conjugated antibody, whereas Ki-67 was detected by a rhodamine-labeled goat anti-mouse antibody. Finally, sections were counterstained by DAPI. The number of cells positive for Ki-67 and TUNEL immunoreactivity was counted per hair bulb and was normalized to the number of total (DAPI+) cells.

Quantitative "real-time" PCR (Q-PCR)

Q-PCR was performed on an ABI PRISM 7000 Sequence Detection System using the 5' nuclease assay. Total RNA was isolated from pools of freshly dissected HFs (n=100–200) using TRIzol (according to the manufacturer's protocol) and were reverse transcribed. PCR amplification was carried out by using TaqMan primers and probes using the TaqMan Universal PCR Master Mix Protocol. As internal controls, transcripts of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were determined. The amount of CB receptor-specific transcripts was normalized to those of GAPDH using the ΔCT method.

Determination of endocannabinoid levels

Freshly isolated HFs weighing ~50 mg were homogenized in Tris buffer containing 7 ng of $^2H_4$-anandamide The homogenate was centrifuged in several steps. The amounts of AEA and 2-AG in the samples were determined using inverse linear regression of standard curves. Values are expressed as fmol or pmol per mg wet tissue.

Solutions

Before each measurement keratinocytes were kept at room temperature for 30 min in normal Tyrode's solution (in mM: 137 NaCl, 5.4 KCl, 0.5 MgCl2, 1.8 CaCl2, 11.8 HEPES-NaOH, 1 g/l glucose, pH 7.4).

The solution was exchanged to a modified Tyrode's solution (TY) where half of the NaCl was replaced by Na-glutamate before a hypotonic challenge. Hypotonic solutions had half or all of the Na-glutamate removed having an osmolarity of approximately 75% or 50% of the original Tyrode's solution, respectively. Calcium-free external solutions had no added calcium and 1 mM EGTA. When hypotonic stress was tested on cell proliferation/ differentiation three different solutions were used. Hypotonic culturing solution (LS50) was prepared by adding distilled water to NS to reach a final osmolarity as for TY50. This, however, decreased the serum content of the medium. As this change alone was suspected to alter the proliferation/differentiation properties of the cells, a low-serum culture medium (LS) was also used as a control. LS was prepared as LS50 but Tyrode's solution was used for supplementing NS instead of distilled water. LS was thus isotonic but it had the same low serum content as LS50.

Patch clamp and conventional microelectrode measurements

Cells were voltage-clamped in the whole-cell mode of the patch clamp technique using an Axopatch 200A amplifier. Briefly, pipettes had resistances of 2–3 MΩ when filled with an artificial internal solution, containing (in mm) 110 K-Aspartate, 20 KCl, 2 $MgCl_2$, 5 EGTA, 5 HEPES and 2 MgATP (pH was set to 7.3 using KOH). In experiments where the involvement of calcium-activated currents was tested an additional 5 mM 1,2-bis(2-aminophenoxy) ethane-N,N,N',N'-tetraacetic acid (BAPTA) was included into the pipette solution to prevent the rise in $[Ca^{2+}]_i$. Passive electrical parameters of the cells were determined with 5 mV pulses of 40 ms duration. The holding potential was set to 40 mV. The linear capacitance of the cells varied between 20 and 40 pF. Electrical signals were digitized at 1 kHz and data were analysed using

the pClamp 6.0.4. Junction potential was calculated by the pClamp 9.0 software using the concentration of different ions and chemicals in the external and pipette solutions. Transmembrane potentials were recorded using glass microelectrodes filled with 3 m KCl and having tip resistance between 25 and 30 MΩ and connected to an Axoclamp- 2B amplifier. Data were digitized at 100 kHz using Digidata 1200 and stored for later analysis.

Assay of [Ca2+]i

Intracellular calcium concentration was determined. Cells were loaded with 5 µM Fura-2 AM, excitation wavelength was altered between 340 and 380 nm, while the emission was monitored at 510 nm. [Ca2+]i levels were calculated from the ratio (R=F340/F380) of the fluorescence intensities using in vivo calibration data.

Determination of cellular proliferation

Investigating the effect of osmotic stress on cell proliferation cells were cultured in 96-well plate using 5000 cell/well starting density. Quadruplicate samples were used for each treatment. In control experiments cells were continuously cultured in NS, LS or LS50 medium throughout 12 days. Every second day cell proliferation was measured by MTT assay. The culturing solution was exchanged on the days prior to these experiments. In the test experiments culturing was started in either NS or LS in triplicates. On the sixth day the culturing of one part of the samples was continued in the same medium, while on the other samples the culturing solution was exchanged to LS and LS50 or NS and LS50, respectively. Cellular proliferation was measured using 3-[4,5-dimethyl- 2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide (MTT assay). The concentration of tetrazolium crystal, as an indicator of the viable cell

number formed from tetrazolium salt in the mitochondria, was determined colorimetrically (A550).

Western blot analysis

Western blot analysis was performed on the 10th day of culturing. Monoclonal mouse primary antibody against the keratinocyte differentiation markers involucrin, filaggrin and transglutaminase were added to the membranes. Peroxidase-conjugated goat anti-mouse IgG antibodies were used as the secondary antibody, and the immunoreactive bands were visualized by an enhanced chemiluminescence Western blotting detection kit on light sensitive films.

Statistical analysis

Statistical analysis was performed using a Mann-Whitney *U* test for unpaired samples as appropriate, was used. A difference was regarded significant when $P < 0.05$ and marked with *.

RESULTS

The role of TRPV1 in the regulation of hair cycle; differences in hair cycle stages between control and TRPV1 knockout mice

The expression of TRPV1 shows hair cycle-dependent differences in mice

      Similar to human epidermis, adolescent wild-type C57BL/6 mouse skin showed strong TRPV1 immunreactivity (IR) on (mostly basal) epidermal keratinocytes. In addition, also similarly to our previous human data, in the hair follicle, TRPV1-IR was exclusively restricted to the epithelial compartments (note the TRPV1-negativity of the dermal papilla during all hair cycle phases). Analysis of depilation-induced hair follicle cycling in these mice, however, revealed discrete, but important and statistically significant changes in the observed specific IR patterns corresponding to TRPV1 protein expression. Intriguingly, the strongest IR signal was detected on keratinocytes of the epithelial strand of the regressing catagen follicle and of the secondary hair germ of telogen hair follicles. With the exception of an asymmetric, disclike region in the anagen VI hair matrix, the most highly proliferating cell populations in the hair follicle epithelium showed a slightly reduced intensity of TRPV1-IR. The inner root sheath and the distal, precortical hair matrix also showed only strongly reduced TRPV1- IR.

Lack of TRPV1 exhibits a significant delay in catagen and telogen transformation

      As TRPV1 activation by capsaicin caused hair follicle regression (anagen– catagen transition) in human hair follicle organ culture, spontaneous hair follicle-cycling was compared between age-matched

TRPV1 wild-type and knockout mice by quantitative histomorphometry. The skin of Trpv-1$^{-/-}$ mice showed no obvious macroscopic or microscopic abnormalities compared to agematched wild-type control. However, on day 19, Trpv-1$^{-/-}$ mice exhibited a significant delay in the first spontaneous transition of their hair follicles from morphogenesis stage 8 (which is often confused with ''the first anagen'') compared to wildtype littermates. This catagen retardation was independently confirmed by cumulative hair cycle score (383716 vs 267726 in wildtype and Trpv-1$^{-/-}$ mice, respectively). Likewise, subsequent telogen development (P25) was slightly but significantly retarded (hair scores of 44979 vs 41273 in wild-type and Trpv-1$^{-/-}$ mice, respectively) in the absence of functional TRPV1 signaling. Instead, the first spontaneous anagen development (P32) and subsequent hair follicle cycling (P45) were not significantly different between Trpv-1-competent and –deficient mice. This suggests that, in murine back skin pelage hair follicles, TRPV1- mediated signaling is important for modulating the transition from the final stages of hair follicle morphogenesis to that of cycling skin appendage, whereas signaling via this receptor looses functional importance once hair follicle cycling has been initiated.

The role of cannabinoids in the reglation of hair follicle biology

AEA, unlike 2-AG, inhibits hair growth

First, microdissected, organ-cultured human scalp HF in the growth stage of the hair cycle (*i.e.*, anagen VI) were stimulated with one of the bestcharacterized endocannabinoids, N-arachidonoylethanolamide (anandamide, AEA). AEA significantly and dose-dependently inhibited hair shaft elongation and (as revealed by determining the number of Ki67 positive cells) hair matrix keratinocyte proliferation. In contrast, the

endocannabinoid significantly stimulated keratinocyte apoptosis in the epithelial hair bulb (as assessed by TUNEL labeling) as well as premature HF entry into apoptosis-driven organ involution (catagen). It is worth noting, however, that AEA did not affect HF pigmentation, since the melanin content of anagen VI HF remained unchanged. We have also investigated the effect of the other main endocannabinoid, 2-arachidonoylglycerol (2-AG). Interestingly, 2-AG did not significantly alter human hair shaft elongation *in vitro*, HF proliferation, apoptosis, or catagen entry.

CB1, but not CB2, is expressed in the HF, and its level is regulated by the hair cycle

By independent immunohistochemical methods, specific CB1 immunoreactivity (CB1-ir) was identified in the HF epithelium, primarily in outer root sheath (ORS) keratinocytes (but not on the fibroblasts of the HF dermal papilla). In addition, transcription of the CB1 gene in freshly isolated, microdissected human scalp HFs (more precisely: anagen VI hair bulbs) was demonstrated by quantitative RT-PCR. In contrast, of great importance, neither immunohistochemistry nor Q-PCR indicated the expression of CB2 in the HF. Intriguingly, CB1 protein expression significantly increased on hair matrix (and, yet only marginally, on ORS) keratinocytes of cultured HF, which had been experimentally induced to undergo premature HF involution (catagen) phase by interferon-γ (IFNγ) treatment. Moreover, we have also found that the intensity of CB1-ir was also up-regulated on AEA-treated catagen HF. These data show that normal human scalp HFs express CB1 (but not CB2) on the gene and protein level, and suggest that the intrafollicular CB1 expression is hair cycle-dependent.

Effects of AEA are mediated by CB1 but not by TRPV1

The above data also support the argument that the effects of AEA on the human scalp HF may be transmitted by CB1 receptors. We found that the specific CB1 antagonist AM-251, which alone did not modify hair shaft elongation, completely abrogated the hair growth-inhibitory effect of AEA and normalized hair growth parameters to the vehicle control level. This finding corroborates the missing evidence of CB2 expression in human scalp HF on either the protein or gene level and suggests that the potent hair growthinhibitory actions of the endocannabinoid AEA are most likely mediated by CB1. However, previous reports have also documented that AEA may also activate TRPV1 and hence may act as an "endovanilloid" substance. Therefore, we also measured the possible role of TRPV1 in mediating the effects of AEA. TRPV1 antagonist iodoresiniferatoxin (I-RTX), which on its own did not modify "basal" hair growth, was unable to prevent the effect of AEA to inhibit hair shaft elongation suggesting the lack of involvement of TRPV1. Further corroborating this statement, we have also found that AEA and the TRPV1 agonist capsaicin exerted similar and, of great importance, additive effects to suppress hair growth, to inhibit the proliferation of HF matrix keratinocytes, and to induce intrafollicular apoptosis. Since the hair growth-inhibitory effect of capsaicin (confirming our previous results) was fully abrogated by the TRPV1 antagonist I-RTX but not affected by the CB1 antagonist AM-251, these findings strongly support the argument that the synergistic endocannabinoid and vanilloid systems operate independently to inhibit human hair growth and hence the effects of AEA are indeed exclusively mediated by CB1.

HF are sources of endocannabinoids

We showed for the first time that freshly dissected HF not only respond to but, intriguingly, also express such endocannabinoids as AEA and 2-AG. However, it was noteworthy to observe that whereas the level of AEA (6.6 –11.2 fmol/mg tissue, range, *n*=2) was comparable to those of, *e.g.*, heart samples (~7.7 fmol/mg tissue), the level of 2-AG was much lower (0.2– 0.3 pmol/mg tissue, range, *n*=2) than previously found in cardiac tissues (~3.5 pmol/mg tissue).

THC also inhibits hair growth

Almost identical to the actions of AEA reported above, THC significantly inhibited hair shaft elongation in a dose-dependent fashion, suppressed proliferation of HF keratinocytes, and induced both hair matrix keratinocyte apoptosis and premature catagen development. These data, therefore, suggest that exocannabinoids can mimic the hair growth-inhibitory effects of endocannabinoids. We also determined the effect of THC on the melanin content of the HF. During this measurement, to differentiate the effect of the exocannabinoids from the wellknown catagen-associated "shut-down" of follicular melanogenesis, the melanin content of only those THC-treated HF were defined, which were not yet transformed to catagen. Interestingly, as opposed to findings with AEA, we found that THC significantly and dose-dependently suppressed the melanin content of the HF, suggesting THC may also exert inhibitory effects on follicular melanogenesis *in situ* (independent of the normal, catagen-associated suppression of the melanin production of the HF).

The role of stretch-activated channels in the regulation of the membranepotential and proliferation of keratinocytes

Hypotonic solutions cause marked hyperpolarization

We first determined whether the application of a mechanical stress would alter the MP of keratinocytes. To apply a reproducible stress, keratinocytes were superfused with hypotonic solutions (TY75 or TY50; see Materials and methods). Measurements were carried out using either conventional or patch-clamp microelectrodes Under these conditions the MP of HaCaT cells was found to be -27 ± 3 mV (n=10) when measured with conventional microelectrodes. The cells had a stable MP in modified Tyrode's solution (TY). Upon the addition of a hypotonic solution (TY75) the MP increased and reached a new, more negative, stable value(-39 ± 7 mV). Further increasing the stress, by applying a more hypotonic solution (TY50), caused further hyperpolarization, which again reached a new stable value (-52 ± 2 mV). Upon returning to TY solution with normal osmolarity the cells depolarized. The recovery, however, was not fully complete within the timeframe of 5 min as the MP was more hyperpolarized (-33 ± 6 mV) compared with control conditions suggesting some long-term effect of the hyposmotic shock. Similar observations were made using the whole-cell mode of the patch clamp technique. The resting MP measured under these conditions in modified Tyrode's solution (-19.7 ± 2.3 mV; n=20) was not significantly different from that reported above. Upon changing the solution to TY50 the cells became hyperpolarized and the MP reached a new value within 5 min. Albeit the time course of the potential change was similar to that measured with conventional microelectrodes, the actual potential change was somewhat smaller, the MP reached only -31.1 ± 2.8 mV.

PKC isoenzymes play a crucial role in the regulation of keratinocyte proliferation and differentiation. We tested therefore if the alteration of PKC might influence the response of HaCaT cells to hypotonic stress. After treating the cells with the general PKC activator phorbol-12-myristate-13- acetate (PMA) overnight, the resting MP of the PMA-treated cells (-20.4 ± 2.8 mV; n=18) was not different statistically from that of the control cells, the application of the hypotonic solution, TY50, brought a larger hyperpolarization, to -38.7 ± 3.4 mV. Not only was the change in MP statistically different between the control and the PMA pretreated cells, but the time course of the potential change in the latter case was also faster.

Membrane currents are altered by hypotonic stress

To describe the currents activated by a hypotonic challenge, membrane currents were measured using the whole-cell mode of the patch clamp technique. Cells were voltageclamped to -40 mV and subjected to a pulse protocol consisting of a brief hyperpolarization to -80 mV and then a 600 ms long depolarization to various MPs. This was repeated following the exchange of solution to TY50. Note that current measurements were carried out on the same cells as the MP measurements described in the previous paragraph. Control and PMA pretreated cells had essentially identical currents in TY solution. Following hypotonic challenge the current became larger with the effect being more pronounced for PMA pretreated cells. To display the voltage dependence of the current (I(Vm)), the difference between the currents at the end of depolarizing pulses and the current at the end of brief hyperpolarizations were calculated for both control and PMA pretreated cells. The hypotonic stress induced an increase in I(Vm) at all voltages tested.

Hypotonic stress-induced hyperpolarization depends on the presence of chloride

To assess the contribution of chloride to the above-described changes in MP and current, the previous experiments were repeated in chloride-free solutions. The removal of chloride slightly but not significantly shifted the resting potential of keratinocytes to depolarized values (to -14.2 ± 2.8 mV; n=6). The addition of chloridefree TY50 caused a hyperpolarization to -19.4 ± 3.9 mV, which was significantly smaller than the change in chloride containing solutions. In line with the above, the removal of chloride essentially eliminated the increase in I(Vm) following the hypotonic challenge at all MPs tested. As it will be demonstrated below the hypotonic challenge induced an elevation in [Ca2+]$_i$. It was, therefore, of interest to see whether the just identified chloride current is calcium activated, or not. To test this hypothesis membrane currents were measured using 5 mm BAPTA in the pipette solution to prevent any increase in [Ca2+]$_i$. I(Vm) under control and hypotonic conditions reveal that the presence of BAPTA did not prevent the hypotonic stress-induced increase in the current. This result indicates that the identified chloride current is likely to flow through mechanosensitive channels rather than through calcium-activated chloride channels.

Hypotonic stress induces an elevation in [Ca2+]$_i$

As the activation of MSCs is often accompanied by a rise in [Ca2+]$_i$ we tested if hypotonic solutions would induce an alteration in [Ca2+]$_i$. The measured changes in [Ca2+]$_i$ could be classified into two groups. Upon the application of the hypotonic solution [Ca2+]$_i$ became elevated, and this elevation was either transient, reaching a definite peak and then declining in the presence of the hypotonic challenge (termed 'Transient'

hereon), or it was prolonged, lasting as long as the hypotonic solution was applied (termed 'Slow' hereon). Transient responses were readily detectable for both TY75 and TY50 solutions with the larger change in tonicity causing a larger elevation in $[Ca2+]_i$ (65 ± 4 nM, n=6 and 91 ± 3 nM, n=5, respectively). On the other hand, 'Slow' responses were only seen when the TY50 solution was applied (the average amplitude was 12.4 ± 4 nM, n=10) whereas TY75 solution failed to induce Slow type changes in $[Ca2+]_i$ on normal HaCaT cells. To see if the calcium transients are similarly affected by PKC phosphorylation as the MP was, the above experiments were repeated on cells that were pretreated with PMA overnight. Hypotonic stress again caused an elevation in $[Ca2+]_i$ and, furthermore, the calcium transients could again be classified as Transient or Slow responses. The amplitude of the 'Transient' calcium responses generated by hypotonic changes was, on average, larger but not significantly different on PMA pretreated than on control cells (70.8 ± 9 nM, n=6 in TY75 and 99.2 ± 9 nM, n=8 in TY50) in contrast with the data obtained in the electrophysiological studies. However, on PMA pretreated cells the TY75 solution was already capable of initiating a detectable Slow type response. The amplitude of 'Slow' responses was 9.9 ± 4 nM, n=5 in TY75 and 14.8 ± 5 nM, n=8 in TY50 solution on PMA-treated keratinocytes. The ATP-evoked calcium transients in control and PMA pretreated cells were also similar both in their amplitude, in their rate of rise, and in their return to the resting level. Although the rate of rise of the hypotonic stress-induced transients was smaller than those evoked by ATP, control and PMA pretreated cells did not differ significantly in this respect. Furthermore, the time constants of the decline of $[Ca2+]_i$ following the hypotonic challenges were similar in control and PMA treated HaCaT cells. These data strongly suggest that the efficacy, but not the amplitude of $\Delta[Ca2+]_i$ caused by a hypotonic

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challenge depends on the pretreatment with PMA. Both on control and on PMA pretreated cells the elevation in $[Ca2+]_i$ induced by the hypotonic stress critically depended on the presence of external calcium. In control condition, the removal of calcium from the external medium significantly suppressed the amplitude of a calcium transient during a hypotonic challenge.

Hypotonic stress alters the proliferation and differentiation of keratinocytes

The proliferation of HaCaT cells if continuously cultured in isotonic solution with low serum content (LS) was continuous, although reduced compared with that of the normal culture medium (NS). Cell differentiation in LS, as assessed by measuring the amount of the differentiation markers involucrin (INV), filaggrin (FIL) or transglutaminase (TG) at day 10, was increased in parallel to the reduced proliferation rate. On the other hand, the hypotonic solution did not induce differentiation, if any it reduced the production of involucrin, filaggrin and transglutaminase, indicating that the cells were, most likely, arrested at a certain phase of the cell cycle. On the other hand, a sudden hypotonic stress was accompanied by an increase in proliferation rate. Cells were cultured in NS then the culture medium was exchanged to LS at day 6. This, due to the decrease in serum decreased the proliferation rate. If the cells were transferred to LS50 instead of LS the suppression of proliferation was markedly reduced. The decreased proliferation seen with the shift from normal to low serum was accompanied by an increase in production of involucrin, filaggrin and transglutaminase. The hypotonic stress not only partially prevented the suppression of cell proliferation, but also decreased the production of the three markers of differentiation when compared with that of LS. If the culture medium was LS from the

start, the hypotonic challenge, just as the change to normal serum at day 6, induced an increase in proliferation. The observations suggest that a sudden hypotonic stress induces the proliferation and suppresses the differentiation of keratinocytes.

DISCUSSION

Differences in the hair cycle between control and TRPV1 knockout mice

Recently we have growing number of evidence about the expression of TRPV1 in the peripheral tissues. We found TRPV1 expression pattern in the mouse very similar to human skin, and the TRPV1-ir was also hair cycle- dependent. In spite of some differences between human and mouse TRPV1 expression pattern, the mouse is a good model for studying the biological properties of the follicles.

The functional role of TRPV1 can be well examined by comparing the control and TRPV1 knockout mice. Our results show that there are differences between the two groups: the development of the $TRPV1^{-/-}$ mice's follicles showed significant delay compared to their wild type littermates. This is great in concordance with our results in human follicles, namely the activation of TRPV1 by capsaicin induces anagen-catagen transformation. These results can be explained by the fact that the stimulation of TRPV1 –by inhibiting the elongation of the follicle ,and inducing apoptosis in the matrix keratinocytes- leads to catagen transformation (which phase can be characterised by regression and activation of apoptotic prcesses). It seems from our results that (at least in case of the TRPV1-mediated processes) examining the mouse skin and follicle is a good model for the human experiments.

Our results have clinical significances, too: pharmacological stimulation of TRPV1 can decrease the proliferation of the keratinocytes and

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follicular cells. Applying different vanilloid agonists may have therapeutic effect on different hair and skin disorders related to increased hair growth, like hirsutism. At the same time applying TRPV1 antagonists may role in the treatement of such disorders, that are related to loss of hair, like alopecia and effluvium.

Role of the cannabinoid system in the biological processes of the human hair follicle

According to the latest results the skin and it's derivatives have not only barrier function but they can be classified into a non-classical neuro-endocrin system, too. One of the evidences of this fact is that the endocannabinoid AEA (which is produced even in the follicles) and the well- known exogen cannabinoid Δ^9-THC influences many function of the human hair follicle. Both molecules- very likely through a CB1-mediated pathway- decreases the elongation of the hairshaft and induces a catagen transformation related to increased apoptosis. By activation of the cannabinoid system the changes in the function of the hair follicle are in line with those experiments where the apoptotic effect of the AEA was shown (e.g. in prostate tissue).

We detected the expression of CB1 both in protein and in mRNA level in the follicle and the expression of CB1 was increased during catagen phase. Our results showed that the human hair follicle is both the source and the target of the endocannabinoid AEA, which can function as a negative regulator of the hair cycle. Interestingly, the concentration of the other endocannabinoid, 2-AG was very low in the follicle. One reason can be the high level of the enzymes participate in the degradation of the molecule.

Earlier studies showed that AEA might exert it's cellular effects via a CB1/CB2 and/or TRPV1 mediated pathway (so it can have both

endocannabinoid and endovanilloid function). Our aim was, therefore, to decide which of them is the target of AEA. The inhibitory effect of AEA on the hairshaft elongation was totally prevented by CB1- specific antagonist AM-251, and at the same time the TRPV1- antagonist did not show that kind of effect. It can be therefore supposed that AEA exerts it's effect exclusively through the CB1. By applying the AEA and the TRPV1 agonist capsaicin at the same time we detected an additive effect, further underlying our hypothesis. One can say that the endogen cannabinoid and vanilloid systems are in close (and "helping") relation, but they regulate independently the biological functions of the follicles. This hypothesis was further strenghtend by the results showing that the morphogenesis of the TRPV1 knockout mice's follicles exhibited significant delay in the catagen transformation compared to their wildtype littermates.

Although our results showed that the follicle is able to produce the endocannabinoids (to make a possibility for the paracrin- autocrin regulation of the follicular functions), it was surprising that the CB1 antagonist AM-251 alone did not affect the hairshaft's elongation. It is known that the level of the endocannabinoids can be changed in case of inflammatory skin disorders. Because it is often accompanied by hair loss, maybe the expression of the endocannabinoid molecules increases in these statements. The CB1 antagonists prevented the elongation-inhibitory effect of the cannabinoids, therefore they can have a significant therapeutical benefit in the treatment of hair loss disorders (maybe used together with TRPV1 antagonists). Combined with vanilloid agonists, in diseases related to increased hair growth, local application of cannabinoids maybe helpful in the treatment of diseases with increased hair growth.

Effect of mechanosensitive channels on the electrophysiological properties, proliferation and differentiation of the keratinocytes

By examining the function of the MSCs', our results showed that decreasing the tonicity of the solution shifted the membrane potential of the cells to a more negative value. Therefore it can be concluded that there are mechanosensitive anion -(influx of negatively charged ions → hyperpolarization) or cation (influx of positively charged ions → depolarisation) channels in the surface of the keratinocytes. Removal of the extracellular chloride significantly decreased the hyperpolarization, therefore the reason of the changes in the mebrane potential maybe the opening of chloride channels.

Other types of the MSCs' are the non-specific cation channels. One part of them are permeable to Ca^{2+}, so the opening of these channels increase the $[Ca^{2+}]_i$. Under Ca^{2+} -free conditions the amplitude of the Ca^{2+} tranzients evoked by hypotonic solutions was decreased, therefore one can suppose that the increase of the $[Ca^{2+}]_i$ is the consequence of the opening of MSCs. We examined whether the chloride current is caused by the elevation of the $[Ca^{2+}]_i$ or not. Because in the presence of BAPTA (a well- known Ca^{2+} chelator) our results showed that there was no significant difference between the control (without BAPTA) and the BAPTA-containing solution, therefore it is likely that the activation of the chloride channels are independent of the $[Ca^{2+}]_i$.

The osmotical stress and the changes in the $[Ca^{2+}]_i$ affects both the proliferation and differentiation of the cells. The PKC isoenzmyes play a significant role in these processes. It can be concluded that the activation of the PKC system enhanced the open probability of the MSCs by phosphorilating them. It induces therefore a bigger chloride/ calcium current in the keratinocytes.

The usage of hypotonic solutions in our experiments is a good modell for the stress applied to the cells, which is a well –known base of the hyperproliferation in the keratinocytes, leading to several diseases. It is rather a sudden hypotonic stress, than the continuous mechanical force increases the viable cell number and decreases the differentiation of the keratinocytes, which can lead even to clinical symptomes. It is in a good concordance with the results from other researches, where it was said that the periodical mechanical effort causes keratinocyte hyperproliferation.

In summery we can conclude that the changes in the osmolarity , as one kind of mechanical stress can result keratinocyte hyperproliferation and de- differentiation. It is caused partly by the opening of the MSCs, which are permeable to chloride, but for the long-term effects also the changes of the $[Ca^{2+}]_i$ can be responsible.

SUMMARY

In our experiments we have investigated the role of different signalling mechanisms in the regulation of the biological properties of the skin and its derivatives. In our work we presented the first evidence for the expression of transient receptor potential vanilloid -1 (TRPV1) in the non- neural cells of mouse skin and follicle. We showed that the expression of TRPV1 in the mouse skin (similar to our previous human datas) depends on the phase of the hair cycle. We concluded that the morphogenesis of the follicles from TRPV1 knockout mice shows a significant delay compared to their wildtype littermates. These results inform us about the inhibitory role of the TRPV1- coupled signalling mechanisms in the regulation of the hair growth.

By studying the cannabinoid system, we presented that the human follicle produces significant amount of anandamide (AEA). It is also of evidence that AEA and the well- known exogen cannabinoid Δ^9-THC –most likely via CB1- decreases the hairshaft elongation and the proliferation of the matrix keratinocytes and induces catagen transformation characterized by increased apoptosis. Furthermore we established that the closely related cannabinoid and vanilloid (TRPV1-coupled) systems act independently (but by helping each other) in the regulation of the biological functions of the follicles.

Finally by studying the regulatory role of the MSCs in human keratinocytes we can conclude that the sudden change of the osmolarity as a kind of mechanical stress causes hyperproliferation and de – differentiation of the keratinocytes. These effects are partly caused by the opening of the chloride- permeable (and PKC regulated) MSCs, but for the long term effects the changes of the $[Ca^{2+}]_i$ can be responsible, too.

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