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THE ROLE OF VANILLOID RECEPTOR-1 (TRPV1) IN THE REGULATION OF BIOLOGICAL PROCESSES OF HUMAN HAIR FOLLICLE

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INTRODUCTION

The hair follicle

The hair follicle (an epidermal „mini-organ”), besides performing its conventional functions such as mechanical protection, sensory function, thermoregulation, social and sexual signaling, possesses a unique role that points far beyond the borders of the biology of the human skin. One of the most important features of the hair follicle is the capability of constant regeneration which is based on multilateral communications between various cell populations of different (epithelial, mesenchymal) origins. During the process a key factor is the sophisticated regulation of the mechanisms of proliferation, differentiation, and apoptosis which is performed by a complex network of growth factors, cytokines, neuropeptides, and adhesion molecules produced within and/or around the hair follicle.

Functional anatomy of the hair follicle

At the first glance, the hair follicle resembles to an onion, in which the inner leaves correspond to the *dermal papilla (DP)* surrounded by the cover layers of the *inner (IRS) and outer (ORS) root sheath keratinocytes*. The “leg” of the onion represents the hair shaft whose layers are formed by keratinocytes located above the DP, whereas its color is determined by the pigment production of the follicular melanocytes. The hair shaft actively grows along with the IRS whose terminally differentiated keratinocyte layers (Henle, Huxley, cuticle) cover and protect the hair shaft.

The ORS, whose cells can be characterized by a slow proliferation rate, establishes the next layer of the hair follicle and possesses a continuous communication and cell exchange with the epidermis. In addition, the ORS supports the hair follicle and shaft with oxygen, nutrients, cells (Langerhans-

cells, keratinocytes), and various mediators that play crucial roles in the regulation of the hair cycle.

The hair cycle

Among the mammalian organs, the hair follicle holds a unique, life-long, periodical alteration in the regression and regeneration processes. This phenomenon is called as the *hair cycle* which can be classified to three characteristic phases.

In the *anagen* phase, the hair follicle is characterized by a machinery of growth and development, and it reaches its maximally matured status. This phase, therefore, is dominated by an intense proliferation, mostly seen in the epithelial cell layers. The growth of the hair shaft occurs continuously along with the altered function of accessory elements (appendages) of the hair follicle. In the *catagen* phase, the growth and development of the hair follicle terminate and regression processes are initiated. In the various layers of keratinocytes, a wide-spread apoptosis takes place whereas the decrease in the cellular number of the DP is due to the outward migration of the cells from the DP. In addition, due to the apoptosis of the melanocytes, the pigment content of the hair follicle is also suppressed. Finally, the last *telogen* phase is actually a preparation period for the next anagen stage.

Regulation of the hair cycle

During hair transplantation experiments, it has long been appreciated that the hair follicle possesses a so called "*internal clock*" which is most probably located in the close vicinity or within the hair follicle. Recent findings strongly suggest that this "internal clock" is the DP itself. It has also been postulated that the hair follicle transformations during cycling are driven by controlled switches in the local signaling milieu.

Several growth factors, cytokines, hormones, and other molecules have been implicated to intervene to the processes of the hair cycle. For example, hepatocyte growth factor (HGF), insulin-like growth factor-I (IGF-I), stem-cell factor (SCF), and vascular endothelial growth factor (VEGF) were shown to prolong the length of anagen. In contrast, fibroblast growth factor-5 (FGF5), interferon-γ (IFN-γ), interleukin-1β (IL-1β), transforming growth factor-β_1 (TGFβ_1), tumor necrosis factor-α (TNF-α), neurotrophin-3 (NT-3), NT-4, and brain-derived neurotrophic factor (BDNF) were described as negative regulators promoting the onset of catagen.

It is important to note the role of TGFβ_2 during the cycle. This growth factor has been originally shown to stimulate follicular development during the morphogenesis of the hair follicle. However, TGFβ_2 plays an opposite role during the hair cycle; i.e., it accelerates the processes of regression (catagen).

Since most of the pathological alterations seen in numerous hair growth disorders are based on malfunctions of the hair cycle (i.e., the "biological clock" of the functional hair follicles "runs" faster or slower than required), the deeper insight to the biology and logic of regulation of the process holds a significant clinical relevance.

The vanilloid (capsaicin) receptor-1 (TRPV1) – Structure of the molecule

The capsaicin receptor is non-specific cationic channel which functions as a one of the central integrator molecules participating in the processes initiated by nociceptive stimuli.

In 1997, the molecular description of the first capsaicin-sensitive specific molecule, the vanilloid (capsaicin) receptor (TRPV1) was published using a rat cDNA library. The rat TRPV1 is a 95 kDa protein consisting of 838 amino acids encoded by 2514 nucleotides. On the basis of its structural features, i.e., the TRPV1 shows marked homology to the TRP (transient receptor potential) protein of the *Drosophila melanogaster*, it belongs to the TRP receptor family.

The common features of these receptors are that they possess 6 transmembrane domains and intracellular N- and C-terminals, and, most probably, they form tetramers in the membrane. It was also shown in numerous structural studies that both the extracellular and intracellular sides of the TRPV1 contain various binding sites for regulatory molecules. Similarly to the native receptor on sensory neurons, the cloned TRPV1 also functions as a non-specific, chiefly Ca^{2+} permeable cation channel.

The TRPV1 can be activated by exogenous and endogenous vanilloids

The first substance that was shown to have an "intimate" relationship with the TRPV1 is capsaicin, the pungent ingredient of hot chili peppers (Capsaicum annuum). The effects of capsaicin were described far before the molecular identification of TRPV1; actually, the investigation of its actions on sensory neurons postulated the existence of a so called "pain receptor".

The cellular effects of capsaicin and related compounds can be well characterized as three consecutive phenomena on the sensory neurons. The first one is the *excitation* (developing right after the application of capsaicin), during which the permeability of the cell membrane increases to (mostly) Ca^{2+} and Na^{+} ions and, therefore, leads to the depolarization of cells. The second characteristic process is the *desensitization,* which can be described as a decreased sensitivity to capsaicin and other (chemical, thermal or mechanical) nociceptive agents. Finally, capsaicin applied at high concentrations and/or for a long duration evokes *neurotoxicity,* which is mainly assigned to the increased intracellular calcium concentration ($[Ca^{2+}]_i$) and to subsequent activation of calcium-dependent proteases.

However, it has also been shown that TRPV1 can not only be activated by exogenous vanilloids but also by various endogenous compounds ("endovanilloids") such as the low-threshold heat (~43^{o}C), the decrease of pH (acidosis), and several inflammatory mediators (bradykinin, extracellular ATP,

arachidonic acid derivatives, leukotriens, products of lipid-peroxidation, etc.). These compounds, on the one hand, may directly activate the receptor by binding to the TRPV1 (e.g., heat, acidosis); or, on the other, upon binding to their mostly metabotropic receptors, may indirectly regulate the function of TRPV1 via the modification of intracellular signal transduction pathways (kinase systems, intracellular messengers). These effects may chiefly decrease the heat threshold of the TRPV1 and thus contribute to the development of thermal/inflammatory hyperalgesia.

The expression of TRPV1 is not restricted to neuronal tissues

Using autoradiography and various functional studies, the receptor was first identified on a distinct subpopulation of primary sensory neurons, whose cell-bodies are located in dorsal root and trigeminal sensory ganglia. These neurons, based on their marked sensitivity to capsaicin and to other vanilloids, are generally referred to as "capsaicin-sensitive neurons". In addition, the presence of TRPV1 was also proven in the dorsal horn (mostly in lamina I.) of the spinal cord. Several binding studies, however, identified the receptor in such central nervous system structures that do not possess obvious, direct connection with the primary sensory neurons. These regions were certain parts of the hypothalamus (where the existence of TRPV1 was implicated in the hypothermic action of capsaicin), cerebellum, cortex, striatum, olfactory bulb, pons, hippocampus, and the thalamus.

One of the most intriguing results of research at the TRPV1 field was, however, the introduction of that the functional expression of TRPV1 is not restricted to neuronal tissues. Bíró and co-workers, for example, successfully induces a TRPV1-specific calcium influx to mast cells and glial cells, which event was very similar that described on sensory neurons. Others have also presented the specific action of capsaicin on such cell types as, for example,

human polymophonuclear cells, lymphocytes, thymocytes, bronchial epithelium, and epithelial, interstitial and smooth muscle cells of the urinary bladder.

In addition, of greatest interest, recent immunohistochemical studies have identified the presence of TRPV1 protein on human epidermis and on cultured epidermal keratinocytes (the results were also confirmed by RT-PCR). It was also shown that the receptor in functionally expressed on these cells since capsaicin and acidic solutions resulted is the TRPV1-specific elevation in $[Ca^{2+}]_i$. Further investigations have moreover presented that, on the epidermal HaCaT keratinocyte cell line, the application of capsaicin increased the expression of cyclooxygenase-2 and the production/release of IL-8 and prostaglandin E2 suggesting that the TRPV1 expressed on epidermal keratinocytes may also participate in the induction and/or regulation of inflammatory mechanisms of the skin.

RATIONALE AND AIMS OF THE STUDY

Those investigations that described the functional presence of TRPV1 (i.e., specific regulation of processes of proliferation, apoptosis, differentiation, cytokine production) of various non-neuronal cells types of neuroectoderm or mesoderm origin (including the epidermal keratinocytes); furthermore, the fact that the TRPV1 agonist capsaicin has long been used in clinical practice in the treatment of various dermatological diseases, strongly argue for that the biological role of TRPV1 points far beyond its previously appreciated sensory one. There is, however, a lack of descriptions in the literature which might clarify the exact functions of TRPV1 signaling in the physiology of the skin and its appendages.

Therefore, in the first descriptive phase of our experiments, we aimed to identify the exact skin localization of TRPV1 in various cellular compartments

of different function and origins, with special emphases on the hair follicle and hair follicle-derived cells (such as ORS keratinocytes). We also intended to evaluate whether the TRPV1 expression pattern in the hair follicle might change in parallel to the events of the hair cycle (e.g., anagen-catagen transformation).

In the next phase, we investigated the functional role of the receptor. We wished to analyze the effects of TRPV1 activation by capsaicin on the fundamental mechanism of the hair follicle (hair shaft elongation) and, furthermore, on such processes as cellular division and proliferation of follicular keratinocytes, differentiation, melanin production, and morphological characteristics. In addition, we also investigated related cellular mechanisms (proliferation differentiation, apoptosis, cytokine production) of human hair follicle-derived cultured ORS keratinocytes. Finally, we intended to elucidate those cellular mechanisms that are initiated by the activation of TRPV1.

MATERIALS AMD METHODS

Isolation and culture of human hair follicles

Human hair follicles were isolated from skin obtained from patients undergoing face-lift surgery. After discarding the epidermis and most of the dermis, hair follicles were separated form the subcutis and then were cultured in supplemented Williams' E medium for 3 or 5 days. Length measurements were performed daily on individual hair follicles then growth/elongation curves were generated. At the end of culturing, the hair follicles were embedded and frozen sections were made. Plastic surgery skin samples were also used for the preparation of frozen or paraffin-embedded full-thickness skin sections.

ORS and HaCaT keratinocyte cultures

ORS keratinocytes were cultured on feeder layer of growth-arrested dermal fibroblasts in a 1:3 mixture of Ham's F12 and serum-free keratinocyte growth media. The ORS cells were obtained from isolated anagen VI follicles by an enzymatic digestion procedure using 0.1% trypsine – 0.2% EDTA. HaCaT keratinocytes were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics.

Histology

A hematoxylin-eosin staining on 8 μm thick cryostat sections was used for studying hair follcile morphology whereas the melanin pigment was visualized by the Masson-Fontana histochemistry.

Immunohistochemistry and -cytochemistry

For the detection of TRPV1 in human skin, an avidin-biotin-complex (ABC) technique was employed. After blocking the samples, sections were first incubated using a primary goat anti-TRPV1 antibody, then by a biotinylated anti-goat secondary antibody, and finally using an ABC reagent. To reveal the peroxidase activity, DAB or VIP SK-4600 was employed as chromogenes. During double immunohistochemistry, after the immunolabeling of TRPV1, dermal mast cells were identified using an anti-tryptase antibody, whereas epidermal (dendritic) Langerhans-cells were stained against the cell-specific marker CD1a.

For the detection of TRPV1 on isolated hair follicles, the tyramide-substrate amplification (TSA) and the alkaline phosphatase activity-based methods were used. For the TSA technique, sections were first incubated using a TRPV1 antibody, then by a secondary multi-link swine antibody, and finally using streptavidin-horseradish peroxidase. The immunoreactions were visualized using TRITC-tyramide.

For the detection of TRPV1 in ORS keratinocytes, a simple immunofluorescence labeling was employed, where incubation with the anti-TRPV1 antibody was followed by a FITC-conjugated secondary antibody.

To evaluate apoptotic cells in co-localization with a proliferation marker Ki-67 in the hair follicles, a Ki-67/TUNEL (Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling) double-staining method was employed. The paraformaldehyde-fixed sections were first incubated by a 7:3 mixture of the reaction buffer and deoxynucleotidyl transferase. Samples were then stained with a mouse anti-Ki-67 antibody, followed by anti-digoxigenin FITC-conjugated antibody which recognizes apoptotic DNA fragments, and finally using a rhodamine-labeled goat anti-mouse IgG.

For simultaneous immunodetection of TRPV1 and NKI/beteb, a sensitive marker of skin melanocytes, sections were first incubated with an anti-TRPV1 antibody and then with a rhodamine-labeled rabbit anti-goat IgG. To visualize the melanocytes, samples were then stained with an anti-NKI/beteb antibody and finally with a FITC-conjugated secondary antibody.

Staining for transforming TGFβ_2 and the differentiation marker filaggrin was carried out with appropriate (anti-TGFβ_2 and -filaggrin) antibodies followed by a FITC-labeled secondary antibody.

Western (immuno) blot analysis

For Western blot analysis of TRPV1 expression, skin extracts or cells were harvested in homogenization buffer, and equal amounts of protein (20-30 μg per lane) were then subjected to SDS-PAGE (on 7.5 % gels) and transferred to nitrocellulose membranes. Membranes were then blocked and probed with the primary anti-TRPV1 antibody overnight. A peroxidase-conjugated secondary antibody was used to visualize the immunoreactive bands by an ECL Western blotting detection kit.

Semi-quantitative reverse-transcriptase PCR (RT-PCR)

For the determination of specific mRNA transcripts for TRPV1 and $TGF\beta_2$, total RNA was isolated from skin samples and hair follicles using Trizol, then reverse transcription was performed using appropriate primers and enzyme mixtures.

Quantitative „real-time" PCR (Q-PCR)

For the quantitative determination of TRPV1-specific mRNA, the 5' nuclease assay was employed. The PCR amplification was carried out using specific TaqMan primers and probes, and the glyceraldehyde 3-phosphate dehydrogenase transcripts were use as internal controls.

Intracellular calcium measurement

Cells were cultured on coverslips and a calcium sensitive probe fura-2 AM was introduced into the intracellular space. The coverslips, containing the fura-2 loaded cells, were then placed on the stage of an inverted fluorescence microscope. Excitation was alternated between 340 and 380 nm using a dual wavelength monochromator and the emission was monitored at 510 nm with a photomultiplier at an acquisition rate of 10 Hz per ratio, and the fluorescence ratio (F_{340}/F_{380}) values were determined. Changes in $[Ca^{2+}]_i$ were calculated using the method of Grynkiewitz and co-workers.

Determination of cellular proliferation

Cellular proliferation was measured by a colorimetric bromo-deoxyuridine (BrDU) assay. The method is based on that the incorporation of the thymidine-analogue BrDU to the DNA of proliferating cells can be colorimetrically measured using a peroxidase-conjugated anti-BrDU antibody and tetramethylbenzidine substrate.

Flow cytometry

The effect of TRPV1 activation on cellular proliferation, differentiation, apoptosis, and the expressions of various regulators of hair follicle growth and development were assessed by flow cytometry. Control and capsaicin treated ORS keratinocytes were immunostained with appropriate primary antibodies and then FITC-conjugated secondary antibodies. The immuno-positive signals (at least 50,000 cells per antigen) were determined using a Coulter EPICS XL-4 instrument and expressed as the percentage of total cell number.

Statistical analysis

Data were expressed as mean ± SEM or mean ± SD, and analyzed using a two-tailed un-paired *t*-test where $p < 0.05$ values were regarded as significant differences.

RESULTS

1) TRPV1 is expressed, both at mRNA and protein levels, on numerous epithelial and mesenchymal cell types of human skin

In the first phase of our experiments – using TRPV1 immunohistochemistry on human scalp skin – we identified a significant TRPV1 immunoreactivity (TRPV1-ir) on epidermal keratinocytes, mostly in the basal layers. We have also found that, besides the keratinocytes, the epidermal CD1a-positive Langerhans-cells also express TRPV1. Furthermore, in addition to sensory nerve fibers (which also served as positive controls), we demonstrated the existence of TRPV1, for the first time, on various cell populations of the dermis. A strong TRPV1-ir was observed on sebocytes and sweat gland epithelium, on endothelial and smooth muscle cells of skin blood vessels, and on dermal smooth muscles. Moreover, double immunohistochemistry revealed that

the tryptase-positive dermal mast cells also express the receptor. The presence of TRPV1 in human skin was also verified by Western blotting and RT-PCR analysis. It is noteworthy that epidermal melanocytes and dermal connective tissue fibroblasts showed no TRPV1-ir.

2) TRPV1 is expressed on several cell types of organ-cultured human hair follicles, as well as on the skin in situ

As revealed by immunohistochemistry on paraffin-embedded sections, TRPV1-ir in human scalp skin *in situ* was also found on several cell layers of the hair follicle. A most prominent signal was detected on ORS and differentiated IRS keratinocytes, and a much less pronounced TRPV1-ir on the hair matrix keratinocytes. The surrounding connective tissue sheath (CTS), as well as the cells of the DP, did not express TRPV1, similarly to the dermal fibroblasts.

An identical tissue expression pattern of TRPV1-ir was found on anagen VI hair follicles cultivated in organ cultures; i.e., most intense signals were localized to ORS and matrix keratinocytes and (yet much less intensively) to IRS keratinocytes. The expression of TRPV1 in the hair follicle was further confirmed by a quantitative detection of TRPV1-specific mRNA transcripts using Q-PCR techniques.

Using NKI/beteb-TRPV1 double immunolabeling on organ-cultured hair follicles, it was also clarified that the follicular melanocytes most probably do not express TRPV1, similarly to the cells of the DP and the CTS.

We have also shown that the above TRPV1 expression pattern was remarkably altered in parallel with the events of the hair cycle. I.e., on interferon-γ-treated catagen hair follicles, the intensity of TRPV1-ir in the ORS keratinocytes was significantly (by $32{,}3 \pm 6{,}8$ %) increased compared to corresponding values of the control (i.e., anagen) follicles.

3) TRPV1 stimulation inhibits hair shaft elongation in specific and dose-dependent manners

The existence of TRPV1 in the human hair follicle raised an obvious question: what is the functional role of the receptor in various physiological processes of the hair follicle? To answer this, using hair follicle organ cultures, we have shown that the TRPV1 agonist capsaicin (applied for 5 days) significantly inhibited hair shaft elongation in a dose-dependent fashion. This action of capsaicin was completely abolished by the specific TRPV1 antagonist iodo-resiniferatoxin (I-RTX), demonstrating a TRPV1-specificity of the observed hair growth-inhibitory effect of capsaicin. These findings indicate the hair growth-inhibitory properties of TRPV1 signaling.

4) TRPV1 activation by capsaicin inhibits hair matrix keratinocyte proliferation and induces apoptosis

Since the activation of TRPV1 resulted in inhibition of hair shaft elongation, we were intrigued to learn whether capsaicin treatment alters such processes of matrix keratinocytes (that play a fundamental role in the establishment of the hair shaft) as proliferation and apoptosis. Using Ki-67/TUNEL double immunohistochemistry on control and capsaicin-treated hair follicles, we evaluated the signs of proliferation and apoptosis in matrix keratinocytes located under the Auberth-line. We have identified numerous proliferating cells in the hair matrix of control hair follicle, with only an insignificant number of apoptotic keratinocytes. In contrast, upon TRPV1 activation, the ratio of proliferating and apoptotic cells was reversed; i.e., in the capsaicin-treated samples, most of the cells were TUNEL positive (reflecting induction of programmed cell death) whereas the number of proliferating keratinocytes was significantly decreased compared to the control hair follicles.

5) TRPV1 stimulation induces morphological alterations showing the characteristics of anagen-catagen transformation

In the next series of experiments, we analyzed the morphological features of capsaicin-treated hair follicles. By classifying the hair follicles to anagen and catagen groups, we found that most of the control (non-treated) hair follicles have shown the morphological characteristics of anagen VI stage. After TRPV1 activation, however, the histological characteristics of the early regression (catagen) phase were observed. I.e., in the capsaicin-treated hair follicles, the DP became thin, the dermal fibroblast migrated downward and transformed to a fusiform shape. In addition, the region of matrix keratinocytes also narrowed and, thus, a thin "bridge-like" cellular layer was developed between the hair shaft and the DP. Finally, the pigment content of the altered hair follicle was markedly decreased.

Since the capsaicin-induced decrease in pigmentation of the hair follicles was accompanied by catagen transformation, we wished to define whether this phenomenon is primarily due to the onset of catagen or to the direct activation of TRPV1. These experiments were carried our on such organ-cultured hair follicles which were not yet transformed to catagen by the application of capsaicin. Using Fontana-Masson histochemistry, we found that there was no significant difference between the melanin content of the control and capsaicin-treated follicles suggesting that the decrease in pigmentation is most probably not due to direct activation of TRPV1.

6) TRPV1 activation does not affect the expression of the differentiation marker filaggrin

The morphological changes and catagen induction initiated by capsaicin administration suggested that the activation of TRPV1 might also evoke differentiation in the follicular keratinocytes. Therefore, on capsaicin-treated hair follicles which were not yet transformed to catagen, we investigated the

effect of TRPV1 activation on the expression of the differentiation marker filaggrin. We have shown that the expression of filaggrin, both in the control and capsaicin-challenged hair follicles, was restricted to the layers of ORS and IRS keratinocytes. Densitometry analysis, however, revealed no significant differences in the filaggrin expression seen in the two groups arguing for that TRV1 signaling most likely does not participate in the regulation of differentiation processes.

7) The hair growth-inhibitory effects of TRPV1 signaling may in part be mediated via TGFβ_2

TGFβ_2 is a negative regulator molecule of the hair cycle and induces very similar morphological alterations in the hair follicle to the above actions of capsaicin. Using immunohistochemistry and RT-PCR techniques, we demonstrated that capsaicin treatment markedly increased the expression of TGFβ_2 not only in the ORS keratinocytes but also in the DP cells and matrix keratinocytes. A neutralizing TGFβ_2 antibody, however, was able to only partially abrogating the capsaicin-induced inhibition of hair shaft elongation indicating that at least some (but, notably, not all) of the hair growth-inhibitory effects of TRPV1 signaling in epithelial biology may be indirectly mediated by stimulation of the expression and secretion of TGFβ_2.

8) TRPV1 is also expressed in cultured ORS keratinocytes as a functional Ca^{2+}-channel

In the next phase of our study, in order to explore the mechanism of action of TRPV1 signaling, we intended to investigate the cellular consequences of the activation of the receptor. These studies were performed on human cultured hair follicle-derived ORS keratinocytes along with human immortalized epidermis-derived HaCaT keratinocytes which were previously shown to express TRPV1. Using Western blotting, immunocytochemistry, and Q-PCR techniques, we have

identified TRPV1 for the first time in the ORS cell cultures at a level comparable to that of positive controls.

Functional calcium image has, furthermore, revealed that capsaicin treatment of ORS keratinocytes – similarly to that seen in HaCaT keratinocytes – elevated $[Ca^{2+}]_i$ in a dose-dependent manner. We have also shown that the action of capsaicin was specific via the activation of the receptor since the TRPV1 antagonist I-RTX prevented the effect of capsaicin to raise $[Ca^{2+}]_i$ on both cell types. These findings strongly suggest that the TRPV1 is expressed as a functional Ca^{2+} channel on ORS keratinocytes as well.

9) TRPV1 stimulation inhibits proliferation and induces apoptosis in cultured ORS and HaCaT keratinocytes

Using a BrdU ELISA method to assess proliferation of ORS and HaCaT keratinocytes, we found that the application of capsaicin, in high-Ca^{2+} (2 mM) culturing medium, significantly suppressed proliferation of ORS keratinocytes in dose-dependent and TRPV1-specific (i.e., inhibited by the antagonist I-RTX) manners. However, as a marked contrast, the TRPV1 agonist exerted a much less growth-inhibitory effect in low-Ca^{2+} (0.4 mM) solution suggesting that the effect of capsaicin to suppress cellular proliferation was chiefly mediated by the concomitant elevation of $[Ca^{2+}]_i$. The negative action of TRPV1 signaling on proliferation of ORS cells was also supported by flow cytometry analyses demonstrating that capsaicin treatment significantly reduced the expression of proliferation markers (such as PCNA, proliferating cell nuclear antigen and Ki-67). Finally, these analyses have also revealed that the activation of TRPV1 by capsaicin remarkably elevated the expression of Annexin-V reflecting the induction of programmed cell death (apoptosis).

10) TRPV1 activation alters the expressions of regulatory molecules of the hair cycle in cultured ORS keratinocytes

In the last period of our study, we investigated the expressions of those cytokines and growth factors which are known as key positive or negative regulators of the hair cycle. We have demonstrated that capsaicin treatment of ORS keratinocytes resulted in a significant decrease in the expressions of positive regulatory molecules such as HGF, IGF-I and SCF, whilst the levels of the negative regulator IL-1β and of the abovementioned $TGF\beta_2$ increased upon TRPV1 stimulation. The expression of other regulatory molecules (FGF-5, IFNγ, TNFα) were not affected by the vanilloid application.

DISCUSSION

1) The expression of TRPV1 in human skin and its appendages

In the first phase of our experiments we successfully identified the expression of TRPV1 on (previously described) epidermal keratinocytes and, for the first time, on epidermal Langerhans-cells. In addition to sensory nerve fibers, we furthermore demonstrated the existence of TRPV1 on various cell populations of the dermis such as sebocytes and sweat gland epithelium, on endothelial and smooth muscle cells of skin blood vessels, and on dermal smooth muscles and mast cells. Similarly to the epidermal keratinocytes, the receptor was also expressed in various keratinocyte populations of the hair follicle (both in the skin *in situ* and on organ-cultured hair follicles) such as ORS, IRS, and matrix keratinocytes. In contrast, TRPV1 was not identified on fibroblasts (dermal, CTS or DP) and melanocytes. It is also noteworthy that the expression of TRPV1 was significantly increased in matrix and ORS keratinocytes of INF-γ-treated catagen follicles suggesting that the cyclic

alterations of the hair follicle may significantly alter the expression profile of TRPV1.

These data unambiguously argue for the expression of TRPV1 on numerous cell types of human skin with distinct origins and functions. Thus TRPV1 joins the group of other receptors (e.g., nicotinic and muscarinic acetylcholine receptors, tachykinin, or glutamate receptors) that were originally described as neuron-specific signal transducers, but later were identified to be functionally active on numerous non-neuronal cell types, including those present in human skin and its appendages.

2) The functional role of TRPV1 in the regulation of biological processes of the human hair follicle

The presented wide-spread expression of TRPV1 in the skin; the different expression levels on keratinocytes with distinct differentiation states; and the increased level of TRPV1 in catagen-transformed hair follicles suggested an obvious question: what might be the function of the receptor in this system?

In hair follicle organ-cultures, we have shown that the specific activation of TRPV1 by capsaicin dose-dependently inhibited the elongation of the hair shaft. In morphological experiments we have, furthermore, found that the inhibition of elongation was paralleled by the development of early catagen stage. These findings suggest that the suppressed growth of the hair shaft was accompanied a complex mechanism of alterations in several cellular compartments of the hair follicle. It has previously been shown that catagen transformation is characterized by the onset of programmed cell death in the keratinocytes and melanocytes. Indeed, capsaicin application resulted in an increased number of TUNEL positive (hence apoptotic) matrix keratinocytes, whereas the number of proliferating (Ki-67 positive) cells was significantly suppressed. These data are in good accord with previous publications describing

that the activation of TRPV1 induces apoptosis in numerous cell types including glial cells, thymocytes, etc.

Our results also suggested that there might be a relationship between the TRPV1 signaling and the TGFβ_2, a negative regulator of the hair cycle. Indeed, capsaicin treatment remarkably elevated the expression of TGFβ_2 in the hair follicles. However, since the neutralizing TGFβ_2 antibody was able to only partially abrogating the capsaicin-induced inhibition of hair shaft elongation, we suppose that although TGFβ_2 might play a role in mediating the effects of TRPV1 activation, the involvement of other signaling pathways should also be encountered. Nevertheless, according to our knowledge, this is the first demonstration of the possible convergence of the TRPV1 and TGFβ_2 signaling mechanisms. Similar phenomena were previously described stating that the activation of TRPV1 on e.g., mast cells, bronchial epithelial cell or keratinocytes results in the increased production and release of such cytokines and growth-modulatory agents as interleukins, prostaglandins, etc.

These observations were further strengthened by our findings on cultured ORS keratinocytes. We have demonstrated that capsaicin treatment of ORS keratinocytes resulted in significant alterations in the expression of cytokines and growth factors that modulate hair follicle growth and cycling. TRPV1 activation suppressed the levels of the positive regulators (HGF, IGF-I, and SCF) whereas the expressions of the negative regulator IL-1β and TGFβ_2 were increased. It appears, therefore, that TRPV1 signaling might function as one of the central integrator molecules regulating the complex biology of the hair follicle by intervening to the cytokine and growth factor production of the hair follicle (keratinocytes) and by modulating proliferation and apoptosis.

We have also investigated the cellular consequences of TRPV1 activation using the above ORS keratinocytes and the epidermal immortalized HaCaT keratinocyte cell line. The TRPV1 protein and the specific mRNA transcripts were successfully identified in the ORS cell cultures at a level comparable to

that of the positive control HaCaT cells. However, TRPV1 was not found on the cultured dermal fibroblasts. This expression pattern was in good accord with those described on the skin *in situ* and on the hair follicles where the existence of TRPV1 was described on various keratinocyte cell layers but not on distinct populations of fibroblasts.

On various neuronal and non-neuronal cell types (including the HaCaT keratinocytes) the TRPV1 functions as a Ca^{2+}-permeable ionic channel. We have shown that capsaicin treatment of ORS keratinocytes elevated $[Ca^{2+}]_i$ in a dose-dependent manner suggesting that the activation of TRPV1 resulted in a Ca^{2+} influx. This was also supported by that removal of extracellular Ca^{2+} as well as the co-administration of the TRPV1 antagonist I-RTX prevented the effect of capsaicin to increase $[Ca^{2+}]_i$. We can conclude, therefore, that a functional TRPV1 is expressed on the ORS keratinocytes as well.

By investigating the cellular proliferation processes, we found that capsaicin treatment effectively decreased the number of proliferating ORS and HaCaT keratinocytes. This effect of capsaicin, however, was not seen when the $[Ca^{2+}]_e$ was decreased suggesting that there might be direct connection between elevation of $[Ca^{2+}]_i$ and the suppression of cellular proliferation after TRPV1 activation. These data, therefore, provided an insight to the possible cellular mechanism of action of capsaicin on ORS and HaCaT keratinocytes, which most probably plays a role in the regulation of biological processes of the intact hair follicles as well.

Interestingly, although the cellular actions affected by TRPV1 activation showed clear calcium-dependence, capsaicin did not significantly affect terminal differentiation of the hair follicle and the cultured ORS keratinocytes, a process that also dependent on $[Ca^{2+}]_e$ in the skin. These data might be explained by several arguments, such as: (1) the induction of catagen and, therefore, the processes of apoptosis (along with the inhibition of proliferation) were initiated earlier by the activation of TRPV1 than possible alterations in the expression of

differentiation markers; (2) the necessarily high calcium concentration of the culturing medium, that was required to maintain the hair follicle in organ-culture, alone induced an increased expression of the differentiation marker filaggrin; hence, the TRPV1-mediated calcium influx was unable to further increase this elevated level; (3) as shown by numerous literature and own data, the effect of capsaicin on various cellular mechanisms possesses a strong dose- and cell type-dependence, as reflected by differential action e.g., on stimulation of cytokine release, cell death, and differentiation; (4) finally, given that capsaicin treatment up-regulates the expression of hair growth inhibitors whereas down-modulates the levels of the hair growth stimulators, it appears that TRPV1 stimulation results in a complex alteration of the cytokine network of the human hair follicle, the net effects of which eventually does not necessarily result in the induction or termination of each cellular processes that might be affected by the elevated $[Ca^{2+}]_i$ following TRPV1 activation.

3) The "double activation" of TRPV1 – Therapeutic implications

Sensory neurons of the skin are in close anatomical contact with the non-neuronal cell types of the tissue such as mast cells, epidermal keratinocytes, and various cell populations of the hair follicle. It was also shown that the excitation of the sensory afferents, by releasing certain neuropeptides (such as e.g., substance P), results in the activation of the neighboring non-neuronal elements (e.g., mast cell degranulation, cytokine release). The released substances then, in turn, further stimulate the sensory endings, hence establishing a bi-directional cellular network (of neuronal and non-neuronal cells) that plays a central regulatory role in physiological and pathological skin mechanisms.

Capsaicin, by activating the above sensory neurons, is widely used in the therapy of chronic inflammation, pain, and itch in numerous dermatological diseases such as prurigo nodularis, notalgia paresthetica, pruritus ani, hemodialysis-related pruritus, uremic pruritus, etc. During these applications,

the investigators suggested that the TRPV1 agonist capsaicin initiates the desensitization of sensory endings and, furthermore, depletes their neuropeptide content; therefore, it "suspends" the above bi-directional neuronal and non-neuronal cellular network.

Our current findings, however, invite an intriguing novel hypothesis that can be called as the "double activation" of TRPV1 with potential therapeutic implications. This theory suggests that the therapeutically applied capsaicin, by co-stimulating the TRPV1-expressing neuronal and non-neuronal cell types of the skin, induces a *simultaneous* release of e.g., neuropeptides, histamine, and inflammatory mediators from the sensory axons and various non-neuronal cells. Since the specific receptors of most of the released agents can be found on most cells types of the skin (including neurons, mast cell, and keratinocytes), the net effect could accelerate the functional performance of the cellular network. With respect to the regulation of the biological processes of the human hair follicle, this might result in e.g., that the sensory neuron-derived substance P (bearing recognized hair growth-modulatory properties) and the TRPV1-expressing mast cells (which are also involved in the regulation of hair follicle growth) appear and/or are activated simultaneously leading to an augmented, further mast cell degranulation both via TRPV1 and certain neuropeptide receptors.

In the framework of our current data and the introduced novel hypothesis, it might be of great importance to thoroughly investigate in the future the non-neuronal expression profile of TRPV1 in various dermatological diseases. This is also supported by that the expression of the TRPV1 was dramatically increased in epidermal keratinocytes of prurigo nodularis patients, a disease whose leading symptom (i.e., itch) and the skin alterations were very effectively normalized by topical capsaicin administration.

Taken together, the current study provides a "hot" new twist to human hair growth control by introducing TRPV1 signaling as a potent, physiologically relevant hair growth-inhibitory force which may be clinically exploited e.g., for

the treatment of unwanted hair growth (hirsutism) by the topical application of TRPV1 agonists, and of hair loss (effluvium, alopecia) by administering TRPV1 antagonists. In addition, our data also invite one to systemically explore in future studies how the anti-proliferative TRPV1 signaling can be manipulated in a clinically desired manner by endogenous and exogenous ligands in the management of hyperproliferative epithelial growth disorders of the skin (e.g., psoriasis, actinic keratosis, keratoacanthoma, and squamous cell carcinoma).

SUMMARY

In the presented study, we investigated the functional role of vanilloid receptor-1 (TRPV1) in the biological processes of human skin and hair follicle. We have shown that TRPV1 is expressed on numerous cell types of human skin (epidermal keratinocytes and Langerhans-cells, dermal sensory neurons, smooth muscle and endothelium of vessels, dermal smooth muscles, mast cells, sebocytes, epithelium of sweat glands). An intense TRPV1 immunoreactivity was also identified on ORS, IRS, and matrix keratinocytes of the hair follicles. A similar expression pattern was observed on organ-cultured anagen VI hair follicles, whereas TRPV1 expression was increased in catagen hair follicles. Functional experiments on organ-cultured hair follicles revealed that the specific activation of TRPV1 by the agonist capsaicin resulted in the inhibition of hair shaft elongation. In addition, we have also shown that this growth-inhibition was accompanied by anagen-catagen transformation of the hair follicle, and by the stimulation of apoptosis and inhibition of cellular proliferation in the matrix keratinocytes. These events, however, were not paralleled by the induction of the differentiation program or changes in the pigment production. We also found that capsaicin treatment elevated the levels of TGFβ_2, a recognized negative regulator of the hair cycle. On cultured ORS keratinocytes, we have demonstrated that the activation of TRPV1 suppressed cellular proliferation and initiated apoptosis, but did not affect the expression of differentiation markers. It was also presented that capsaicin markedly altered the levels of numerous modulators of the hair cycle (expressions of positive regulators were decreased, whilst the levels of negative factors were elevated). We have also shown that these cellular effects were most probably mediated by the TRPV1-dependent elevation of $[Ca^{2+}]_i$. In conclusion, our findings strongly argue for the central role of TRPV1 signaling in the regulation of biological processes of the human hair follicle.

PUBLICATIONS

THE THESIS WAS BUILT ON THE FOLLOWING PUBLICATIONS:

1) Bodó E, *Kovács I, Telek A, Varga A, Paus R, Kovács L, Bíró T*: Vanilloid receptor-1 (VR1) is widely expressed on various epithelial and mesenchymal cell types of human skin. *J. Invest. Dermatol.* 2004; **123(2)**:410-413. **IF: 4,194**

2) Bodó E, *Bíró T, Telek A, Czifra G, Griger Z, Tóth IB, Mescalchin A, Ito T, Bettermann A, Pertile P, Kovács L, Paus R*: A "hot" new twist to hair biology – Involvement of vanilloid receptor-1 (VR1/TRPV1) signaling in human hair growth control. *Am. J. Pathol.* 2004; ***(in press)*** **IF: 6,946**

OTHER PUBLICATIONS:

3) *Papp H, Czifra G,* ***Bodó E****, Lázár J, Kovács I, Aleksza M, Juhász I, Ács P, Sipka S, Kovács L, Blumberg PM, Bíró T:* Opposite roles of protein kinase C isoforms in proliferation, differentiation, apoptosis, and tumorigenicity of human HaCaT keratinocytes. *Cell. Mol. Life. Sci.* 2004; **61(9)**:1095-1105. **IF: 4,995**

4) *Conrad F, Ohnemus U,* ***Bodó E****, Bettermann A, Paus R:* Estrogens and human scalp hair growth-still more questions than answers. *J. Invest. Dermatol.* 2004; **122(3)**:840-842. **IF: 4,194**

5) *Conrad F, Ohnemus U,* ***Bodó E****, Bíró T, Tychsen B, Bosio A, Gerstmayer B, Schmidt-Rose T, Altgilbers S, Bettermann A, Meyer W, Paus R*: Substantial sex-dependent differences in the response of human scalp hair follicles to estrogen stimulation in vitro advocate gender-tailored mangement of female versus male pattern balding. *J. Invest. Dermatol. Symp. Proc.* 2004; ***(in press)*****; IF: 0,867**

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1) *Bíró T, Papp H,* ***Bodó E****, Lázár J, Czifra G, Kovács L.* Modification of protein kinase C alters proliferation and differentiation of immortalized human keratinocytes. *Rev. Oncol.* 2002; **4(1)**:82

2) *Gönczi M, Papp H,* ***Bodó E****, Kovács L, Bíró T, Csernoch L.* Effects of recombinant overexpression of PKCα and δ on receptor-coupled calcium handling in HaCaT keratinocytes. *J. Invest. Dermatol.* 2002; **119(3)**:746

3) *Papp H,* ***Bodó E****, Lázár J, Czifra G, Kovács L, Bíró T.* Effects of pharmacological modification of protein kinase C on proliferation and differentiation of human HaCaT keratinocytes. *Acta Physiol. Hung.* 2002; **89(1-3)**:348

4) *Bíró T, Papp H*, ***Bodó E****, Lázár J, Czifra G, Kovács I, Gáspár K, Juhász I, Kovács L.* Opposite roles of protein kinase C isoforms in regulating human HaCaT keratinocyte proliferation, differentiation, and tumor genesis. *J. Invest. Dermatol.* 2003; **121(1)**:218

5) *Bíró T, Papp H*, ***Bodó E****, Lázár J, Czifra G, Kovács I, Gáspár K, Juhász I, Kovács L.* Opposite roles of protein kinase C isoforms in regulating human HaCaT keratinocyte proliferation, differentiation, and tumor genesis. *J. Dermatol. Sci.* 2003; **32(2)**:A169

6) ***Bodó E****, Géczy T, Lázár J, Kovács I, Czifra G, Bettermann A, Kovács L, Paus R, Bíró T.* The cutaneous vanilloid receptor 1 expression suggests multiple functions beyond sensory nerve signaling. *J. Invest. Dermatol.* 2003; **121(1)**:218, No. 823

7) ***Bodó E****, Géczy T, Lázár J, Kovács I, Czifra G, Bettermann A, Kovács L, Paus R, Bíró T.* The cutaneous vanilloid receptor 1 expression suggests multiple functions beyond sensory nerve signaling. *J. Dermatol. Sci.* 2003; **32(2)**: 823

8) *Bíró T, Czifra G,* ***Bodó E****, Lázár J, Tóth IB, Papp H, Kovács I, Juhász I, Kovács L.* Cell and isoform specific roles of protein kinase C isoenzymes in regulating in vitro and in vivo proliferation of keratinocytes and skeletal muscle cells. *J. Invest. Dermatol.* 2004; **123(2)**:A21

9) *Tychsen B, Conrad F,* ***Bodó E****, Kobayashi H, Bíró T, Paus R.* Cyclosporin A Induces Anagen and Increases the Number of Dermal Papilla Fibroblasts in Nude(Foxn1nu/nu) Mice. *J. Invest. Dermatol.* 2004; **123(2)**:A76

10) *Paus R, Ito N, Ito T, Peters E,* ***Bodó E****, Liotiri S, Bíró T, Arck P.* Cutaneous neuroimmunology – lessons from the hair follicle. *Exp. Dermatol.* 2004 ***(in press)***

11) ***Bodó E****, Bíró T****,*** *Telek A, Czifra G, Griger Z, Tóth IB, Lázár J, Mescalchin A, Ito T, Bettermann A, Pertile P, Kovács L, Paus R.* A "hot twist" to hair biology – Involvement of vanilloid receptor-1 (VR1) signalling in human hair growth control. *Exp. Dermatol.* 2004 ***(in press)***