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

ROLE OF TRANSIENT RECEPTOR POTENTIAL VANILLOID-1 (TRPV1) IN THE REGULATION OF BIOLOGICAL PROCESSES OF HUMAN SEBOCYTES AND DENDRITIC CELLS

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INTRODUCTION

Capsaicin-sensitive neurons – cellular effects of capsaicin

Capsaicin is the pungent ingredient of chili peppers (Capsaicum annum) that is responsible for their „hot" taste. The therapeutic uses of capsaicin are widespread, there have reports of it's beneficial effect in diverse pain syndromes, pruritus, psoriasis, cluster headaches, detrusor hyperreflexia and rhinopathia. Capsaicin was also able to inhibit carcino- and mutagenesis in several *in vitro* experiments as well.

Capsaicin is an alkaloid, which also carries the phenol-derived vanillil group. Other vanilloids were also shown to mimic the effects of capsaicin, for example rezinoferatoxin (RTX), considered the ultrapotent analog of capsaicin, which was isolated from *Euphorbia resinifera* a plant belonging to the spurge family.

Capsaicn's mode of action was first described on sensory neurons. It has been known since the 1960s that primary sensory neurons also have efferent functions, which can be blocked with the preapplication of capsaicin. Primary sensory neurons located in the dorsal root (DRG) and trigenimal ganglions show selective suspectibilty to capsaicin. These „capsaicin-sensitive" neurons are mainly characterized by small cell bodies and thin (C-type) axons.

The cellular effects of capsaicin and related vanilloid compounds show three distinct phases. The first phase after capsaicin dosage is excitation; capsaicin increases the permeability of Ca^{2+} and Na^{+} which leads to depolarization. This is followed by both homolog and heterolog desensitation, in other words the cells become insensitive to both vanilloids and other painful stimuli. Finally if capsaicin is given in high concentrations for a long enough time cytotoxicity can be elicited, which can be attributed mostly to an increase

in the intracellular calcium concentration and the subsequent increase in the function of calcium-dependent proteases.

The target of capsaicin: the vanilloid receptor-1 (TRPV1)

The functional target of capsaicin has proven to be a non-specific, mainly Ca^{2+}-permeable cation-channel. By the end of the 1990s it became clear that this capsaicin receptor, or vanilloid receptor-1 belongs to the vanilloid family transient receptor potential (TRPV) ion channels and as such is known as transient receptor potential receptor 1 (TRPV1) in the literature. The members of the TRPV family can be activated by diverse plant-derived alkaloids, but so far TRPV1 is the only receptor sensitive to capsaicin.

The first (rat) TRPV1 channel to be characterized is compromised of 838 amino acids, has a molecular weight of 95 kDa and is coded by 2514 nucleotides. The cloned receptor is functionaly a non-specific, mainly calcium permeable cation channel (P_{Ca}/P_{Na} approximately 10). The receptor is compromised of 6 transmembrane domains, with both N-, and C-terminals located intracellularly, where the vanilloid binding site is also located. The receptor forms functional channels in tetramers, with the fifth and sixth loops of each chain forming the channel's pore. TRPV1 also has many other binding and regulatory sites, for example the three ankyrin repeat domains on the intracellular N-terminal section which serve as protein kinase A phosphorilation sites, or the extracellular allosteric modulational sites. All of these provide an opportunity for numerous agents to influence the channel's function.

The endogenous activation of TRPV1

Besides exogenous vanilloid substances TRPV1 can also be activated by numerous other substances that are created in the organism, most of which play an important role in the origin of pain sensation. The most important

endogenous activator („ligand”) of TRPV1 is the increase of temperature (~43°C) and the decrease in pH (acidosis, pH 5,5). Acidotic pH can not activate any other TRPV channels, but TRPV2-4 channels also show thermosensitivity.

Besides these stimuli other substances, mainly inflammatory mediators (e. g. bradykinin, intra- and extracellular ATP, arachidonic acid derivatives, leukotrienes, end products of lipid peroxidation, etc.) are capable of positively activating TPRV1. These mediators mostly bind to their own (mainly metabotrope) receptors and influence intracellular pathways (kinase-systems, intracellular messengers) to ultimately modify the regulatory sites of TRPV1 and in doing so sensitize the channel by decreasing it's activational threshold. Sensitization might also be affected by the reorginization of lipid rafts surrounding TRPV1, for example the degrading of phosphoinozitol-bi-phosphate (PIP_2) by phospholipase-C (PLC).

Besides indirect senzitization some lipid mediators can also activate TRPV1 directly, for example anandamide, origanaly described as an endogenous cannabinoid, N-arachidonil-dopamine or various lipoxygenase products.

The role of neuronal TRPV1

Agents sensizitizing TRPV1 in neural cells can decrease the receptor's thermal activational threshold, so that TRPV1 can open in physiological temperatures and ellicit pain sensation (thermal hyperalgesia). Based on the above TRPV1 can be considered a central integrator molecule of diverse (e.g.: chemical and thermal) nociceptive stimuli.

Outside of sensory functions primary sensory neurons also have efferent functions. After activation neuropeptides (e.g.: substance-P (SP), calcitonin gene-related peptide (CGRP)) are released locally. These have an effect on the non-neural cells of the innervated organ (mast cells, macrophages, lymphocytes,

etc.) and partake in local trophic, vasoregulatory and immunmodulatory responses. The pathological, cascade-like intensification of these processes can lead to neurogenic inflammation. As such the activation of TRPV1 can lead to neurogenic inflammation, but repeated activation of the cells quickly depletes their neuropeptide reserve and leads to desensitization which can block inflammatory processes. The pathological increase in the release of neuropeptides can contribute to various clinical syndromes that are characterized by the derailment of normal inflammatory adaptation (asthma bronchiale, colitis ulcerosa, interstitial cystitis, arthritis, etc.), and so the pharmacological manipulation of TRPV1 might have beneficial therapeutic effects in these cases.

Activation of TRPV1 sensory efferent terminals also release somatostatin into the bloodstream, which has systemic antiinflammatory and analgesic effects, by blocking the efferent function of sensory terminals. The somatostatin released from the anterior hepatic plexus because of TRPV1 activation presumbly also has an insulin-sensitizing effect, however the elimination of TRPV1 positive sensory fibers from the pancreas increases glucose tolerance.

TRPV1 can also play a role in thermoregulation. While acute systemic application of capsaicin elicits hypothermia, antagonists of TPRV1 increase body temperature, however the pathway of this effect is still unclear. While TRPV1 expressed on peripherial sensory neurons effect thermoregulation, capsaicin delivered directly into the hypothalamus can also cause hypothermia.

Capsaicin administered directly into different parts of the central nervous system ellicits a biological response and typcial morphological changes. Using [^{3}H]RTX specific, high affinity binding could be detected in diverse structures of the brain. The presence of TRPV1 could be provenm in the dorsal root of the spinal cord, the pons, the limbical areas, the cortex,the striatum, and also in several parts of the thalamus and hypothalamus.

The expression and function of TRPV1 on non-neural cells

TRPV1 specific mRNA was found in the kidney at the time of the first cloning of the receptor, and many other tissues and cell types have been proven to express TRPV1 since then, e.g. the smooth muscle-, epithalial- and interstitial cells of the human urinary bladder.

The presence of TRPV1 has also been proven on the epithelial cells of the stomach, and it has been also been demonstrated that it's activation can block cell death caused by alcohol and low pH. TRPV1 is also expressed in the airways; the application of capsaicin causes the smooth muscle cells to contract, while on epithelial cells an influx of Ca^{2+}, release of proinflammatory citokines (IL-6, IL-8 and TNFα) and finally the apoptosis of the cells could be observed.

The activation of TRPV1 on C6 glioma cells and thymocytes increases the Ca^{2+} level of the cells and can induce apoptosis. TRPV1 was also found on microglia cells, where it also caused cell death through mitochondrial damage caused by the increase of intracellular Ca^{2+} concentration.

Capsaicin can also cause apoptosis in tumorously transformed cells. This was proven on the gliablastoma -derived C6 glioma cell line, human cervical cancer-derived HeLa cells, the liver tumor-derived SK-Hep-1 cell line, and breast cancer-derived MCF-7 cells, though the role of TRPV1 was not shown in either of the above cases. Although prostate tumor-derived LNCaP and PC-3 cell lines express functional TRPV1 and capsaicin can cause apoptosis in these cells, this effect is presumably independent of TRPV1. These findings suggest that vanilloid compounds can cause apoptosis independent of TRPV1 signalling.

Numerous papers have reported the effect of capsaicin on immunocompetent cells. Capsaicin causes SP release on human monocytes, macrophages and lymphocytes, and increases the migration of human polymorphonuclear cells. The presence of functional TRPV1 was shown on mast cells, where capsaicin caused interleukin-4 (IL-4) release. TRPV1 expression was also proven on neutrophil granulocytes and the lymphocyte-rich

mononuclear fraction of whole blood. Capsaicin treatment of murine macrophages induced with bacterial lipopolysaccharide (LPS) blocked the cells' PGE_2 synthesis and the inducible nitrogenmonoxide-synthase (iNOS), and also resulted in the inactivation of the transcription factor NF-κB.

The sebaceus gland and sebocytes

The sebaceus gland (glandula sebacea) is a holocrine gland located in the dermis of the skin, where it is primarily associated with hair follicles. It's cells are the sebocytes, and their main function is the production of sebum (tallow). Sebum is mainly made up of neutral lipids, with a relatively high amount of triglicerides, free fatty acids, wax esters, cholesterin and squalene. In animals it plays important roles in the impregnation of fur and thermal insulation, while in some species the sebaceus gland has specialized to produce pheromones. Since these functions are mostly unrecognizable it has been a long-standing view that the human sebaceus gland is an evolutional relic. Recent findings show however, that sebocytes' function goes beyond the formation of the passive barrier function of the skin. Through numerous paracrine, endocrine and immunological functions the sebaceus gland greatly contributes to the normal homeostatic function of the skin.

In vitro sebocyte models have greatly contributed to elucidating the function of sebaceus glands. Since primary cultures have limited sustainability researchers have benefited greatly from cell lines derived from sebocytes, for example the SZ95 cell line. *In vitro* models allow deeper insight into the molecular regulation of sebocytes.

It has been known for a long time that androgens increase the production of sebum. Sebocytes also have the enzyme apparatus to produce steroid hormones, and they are capable of metabolising androgens. For androgens to exert their effects the peroxisome proliferator activated receptors (PPARs) are required. Since PPARs play a central role in the regulation of lipid metabolism in many

tissues, they are also key players in the control of sebocytes by increasing the terminal differentiation and subsequent lipid synthesis of sebocytes. This differentiation can also be induced by arachidonic acid, which causes increased lipid accumalation and apoptosis in the cells. Growth hormone, insulin, and IGF-1 have all been shown to have roles in the proliferation, differantiation and lipid synthesis, but corticotropin releasing hormone (CRH), α-melanocyte stimulating hormone (αMSH) and β-endorphine of the hypothalamus-hypophysis system have also been show to increase lipid synthesis through their receptors.

Sebocytes also participate in the regulation of immunological function and inflammatory processes. They are capable of producing different cytokines (IL-6, IL-1β, TNFα) and lipid-derived inflammatory mediators (LTB4, PGE2) the former of which can also be regulated by PPARs. They are also capable of producing antibacterial peptides after treatment with bacterial inductors, which also causes an increase in proinflammatory TNFα and IL-8/CXCL8 production. SP also increasese the level of proinflammatory citokines, as well as the differentiation and proliferation of sebaceus glands.

Dendritic cells

Dendritice cells are bone marrow derived professional antigen presenting cells, that partake mostly in the initiation of the specific immune response. They are present in the connective tissue of most organs, as well as the T-cell rich regions of lymph nodes and the spleen. Their monocyte-like precursors can be found in a very small fraction in the bloodstream, while more can be isolated from the surface of mucosal barriers and as Langerhans cells in the skin. In this differentiation state they can internalize pathogens, membrane fragments or dissolved proteins via pinocytosis or phagocytosis. This is followed by activation and migration to lymph nodes, where they come into contact with both T- and B-cells. During this process the cells go through maturational

processes and subsequently lose their ability to internalize antigens, however the expression of the major histocompatibility complex (MHC) is increased.

Immature dendritic cells are capable of recognizing numerous pathogen specific molecular patterns through so-called Pattern Recognition Receptors, for example Toll-like receptors (TLR). Their activation initiates the maturation of dendritic cells and leads to the developement antigen-specific immune responses. Another example of PRRs are the mostly dendritic cell-specific C-type lectins (e.g.: CD205, CD206/mannose receptor, CD207/langerin or CD209/DC-SIGN – the dendritic cell specific ICAM-3 binding nonintegrin), which partake in the initiation of receptor-mediated endocytosis.

After antigen uptake the dendritic cells migrate into the lymph nodes. Several chemokines play an important role in this process. Migration can be initiated with e.g. CCL19/CCL21 (chemokine C-C motif ligand 19 and 21) chemokines through CCR7 (chemokine C-C motif receptor 7) chemokinereceptor. During migration the cells undergo maturational processes, the expression of MHC-II molecules is upregulated as are other maturational markers (e.g.: CD86). Dendritic cells can activate cytotoxic (T_c) and helper (T_h) T-cells and also B cells. Antigen presenting cells also express costimulatory molecules such as CD40, CD80, CD86 which partake in the formation of the immune synapse with MHC complexes. Besides direct cell-cell interaction dendritic cells can also influence the developing immune response by producing a wide array of pro- and anti-inflammatory citokines (e.g.. IL-1β, IL-6, IL-10, IL-12, TNFα, etc.).

Based on their origin dendritic cells can belong into one of two populations: myeloid dendritic cells and plasmacitoid dendritic cells, which care characterized by lymphoid markers. In our present study we examined the function of TRPV1 on myeloid dendritic cells.

Myeloid dendritic cells are derived from CD34 positive precursors and express myeloid markers as well (e.g.: CD11b, CD11c, CD13, CD33, mannose

receptors). Both epidermal Langerhans cells and intersticial dendritic cells belong to this lineage, Myeloid dendritic cells show a high degree of heterogenity, which can also be influenced by the activating signals. Dendritic cells activated by bacterial (LPS) or T-cell signals stimulate the induction of T helper-1 (T_h1) cells and subsequently the developement of cellular immune response, while certain antiinflammatory molecules (e.g.: TGFβ, IL-10, PGE_2, steroids) block T_h1 induction and support the formation of T_h2 cells.

Dendritic cells differntiated *in vitro* from monocytes that were isolated from periphereal blood and cultured in the presence of GM-CSF and IL-4 are one of the most widely used models of dendritic cells. Monocyte-derived dendritic cells also respond differently to different activational signals. Activated in the presence of interferon-γ (IFNγ) they mature into dendritic cells that produce IL-12 and support the formation of T_h1 cells, however if the activation happens with PGE_2 T_h2 formation is favored. PGE2 also plays a role in the expression of CCR7 and the regulation of cell migration.

The role of TRPV1 in skin biology – results of previous research

Capsaicin is used world-wide for the mitigation of various dermal and subdermal pain. This positive effect is classicly explained by the acute activation and subsequent desensitation and change in neuropeptide release of C-fibers. Topical application of capsaicin has been tested in numerous neuropathic pain syndromes, but clinical trials have also demonstrated their effectiveness in diseases characterized with itch.

While therapeutic capsaicin's beneficial effect is classicly explained by the compound's effect on neural elements, the most recent models call attention to the role of non-neural cell types. TRPV1 functioning as a Ca^{2+} channel was found on keratinocytes, and capsaicin increased the expression of ciklooxigenase-2 (COX-2) as well as the release of IL-8 and PGE_2. TRPV1 has been show to be expressed *in situ* on hair follicles, it's outer and inner root

sheath keratinocytes and matrix keratinocytes, sweat glands, the endothelium of veins, Langerhans cells and the sebaceus glands of the human skin. The activation of the receptor on human hair follicles blocks their proliferation and induces apoptosis while causing a shift towards the catagen phase.

Although we have more and more information on the role of TRPV1 on the regulation of the skin function, we still have large holes in our knowledge concerning other cell types. Although our previous research showed the importance of the receptor on the biology of hair follicles, the other member of the pilo-sebaceus unit, we have no functional results regarding the sebaceus gland outside of the the *in situ* expression of TRPV1.

Data on the role of TRPV1 on dendritic cells is both minimal and controversial. TRPV1 expression was proven in murine dendritic cells, and it was reported that capsaicin induces the maturation and migration of the cells, which effect could not be proven on TRPV1 knock-out mice. In another study no expression of TRPV1 was found. We know even less about human dendritic cells. In our previous works we have shown the *in situ* expression of TRPV1 on Langerhans cells, but we have no information on the role of the receptor on human dendritic cells.

RATIONALE AND AIMS OF THE STUDY

The aim of the study was to invetigate the expression and functional role of TRPV1 in human sebaceous gland and dendritic cells modells *in vitro*.

To invetigate the sebaceous cells, we used the human immortalized SZ95 sebocyte celline. We addressed the questions below:

1. Is the TRPV1 expressed also on SZ95 sebocytes,as it was reported on human sebaceous glands *in situ?*

2. Can the pharmacological activation of TRPV1 by capsaicin influence the biological processes – e.g. differentiation (lipid synthesis), viability and proliferation) – of SZ95 sebocytes?
3. Does the TRPV1 have any effect on immunological processes and mediator release of sebocytes?
4. Is the TRPV1 really involved in the effect of capsaicin?
5. Which signaling pathways/alterations of gene expression can mediate the effect of TRPV1 activation?

To investigate the human dendritic cells we used monocyte-derived dendritic cells differentiated in prescence of GM-CSF and IL-4 *in vitro*. We were looking for answers for questions below:

1. Is the TRPV1 expressed on human monocytes and monocyte-derived immatured dendritic cells? Does the expression level of TRPV1 change during differentiation of dendritic cells? Is the expression functional, can the TRPV1 work as a Ca^{2+} permeable channel?
2. Does the TRPV1 have any role int he differentiation of monocytes-derived dendritic cells? Can the activation by capsaicin influence the differentiation?
3. How does the activation of TRPV1 influence the viability of dendritic cells?
4. Can the activation of TRPV1 influence the function of differentiated, immature dendritic cells? Is it able to induce activation and maturation of dendritic cells?
5. Can the pharmacological activation of TRPV1 influence the dendritic cell maturation induced by proinflammatory cytokines?

MATERIALS AND METHODS

Materials

We have prepared capsaicin, capsazepine, iodo-resiniferatoxin and arachidonic acid stock solutions with 1000-fold concentration compared to the final used in treatments. These stocks were 1000-times diluted befor treatment to keep the solvent concentration at 0.1 %.

Culturing of SZ95 sebocytes

Human sebaceous gland-derived SZ95 sebocytes were cultured in Sebomed basal medium supplemented with fetal bovine serum, 1mM $CaCl_2$, human epidermal growth factor and antibiotics. The final Ca^{2+} concentration of the medium was approximately 1.25 mM (normal or high-Ca^{2+} containing medium). The low-Ca^{2+} containing medium was not supplemented with Ca^{2+} to set the Ca^{2+} concentration to 0.25mM.

Isolatio of monocytes from human periferial blood

Trombocyte-free human blood was centrifuged on ficoll gradient and monocytes were isolated from buffy coats by immunomagnetic cell separation using anti-CD14-conjugated microbeads.

Differentiaition of dendritic cells

To induce the differentiation of immature dendritic cells, monocytes ($2x10^6$ cells/ml) were cultured in serum-free AIMV medium supplemented with GM-CSF and IL-4. At day 2, the same amount of GM-CSF and IL-4 was added and the cells were cultured for another 3 days. Immature dendritic cells were differentiated at day 5, which was evidenced by specific cellsufrace markers shown by flow cytometry. During the differentiation, cells were treated by daily aplication of capsaicin, capsazepine or combination of the above substances.

Maturation and actvation of dendritic cells

To generate matured dendritic cells, imatured dendritic cells were activated for 24 h with a "pro-inflammatory cytokine cocktail" containing 80 ng/ml GM-CSF, 10 ng/ml TNF-α, 5 ng/ml IL-1β, 20 ng/ml IL-6, and 1 μg/ml PGE_2.

Phenotypic characterization by flow cytometry

Phenotypic characterization of SZ95 sebocytes and monocyte-derived dendritic cells was performed using flow cytometry by determining the forward scatter (size) and side scatter (granulation) values.

Determination of cell surface markers by flow cytometry

To detect the expression of cell surface markers, monocytes and dendritic cells were staind by different fluorochrome-conjugated antibodies: anti-CD83-FITC (FITC-conjugated anti-CD83 antibody) anti-CD14-PE (phycoerithrin-conjugated anti-CD14 antibody) anti-CD209/DC-SIGN-FITC, anti-CCR7-PE and corresponding isotype controls. Fluorescence was detected by a FACSCalibur flow cytometer, data were analysed by using the WinMDI software.

Determination of apoptosis by flow cytometry

Late apoptotic events were assessed also by flow cytometry. Following a 2-day-long treatment with various agents cells were harvested and stained with an Annexin-V-FITC/Propidium Iodide Apoptosis Kit. Fluorescence intensity was measured by a Coulter Epics XL flow cytometer.

RNA isolation, reverse transcription, quantitative real-time PCR

Total RNA was isolated using TRIzol and then 3 μg of total RNA were reverse transcribed into cDNA by using AMV reverse transcriptase and random

primers. Quantitative real-time PCR (Q-PCR) amplification was performed by using specific TaqMan primers and probes and the TaqMan Universal PCR Master Mix Protocol. Realtive gene expressions were determined using by the ΔCT method.

Immunocytochemistry

SZ95 sebocytes were seeded and cultured on sterile coverslips to 60% of confluence. Monocytes were seeded also on sterile coverslips and following the above mentioned differentiation protocol, we gain the adherent immatured dendritic cells at day 5. Cells were fixed, permeabilized, washed and then incubated with the anti-TRPV1 primary antibody. For fluorescence staining, cells were then incubated with Texas Red- or FITC-conjugated secondary antibodies. For light microscopy, cells were incubated by a horseradish peroxidase-polymerconjugated secondary antibody and developed by diaminobenzydine

Immunohistochemistry

Immunhistochemical sudies were performed on formaldehyde-fixed sections of normal human skin samples obtained during plastic surgery and embedded in paraffin. To detect TRPV1, a streptavidine-biotin-complex three-step immunohistochemical technique was employed. Following the inhibition of endogenous peroxidases and blocking the nonspecific binding sites, sections were first incubated with an anti-TRPV1 primary antibody, then with a biotincoupled secondary antibody and, finally, with streptavidine conjugated with horseradish peroxidase. To reveal the peroxidase activity, DAB was employed as a chromogene.

Western blotting

Cells were harvested in lysis buffer and homogenized on ice using ultrasonic homogenizator. Samples were prepared adding ß-mercaptoethanol and SDS, and then proteins were denatureted by boiling. Samples containing equal amount of proteins were subjected to SDS–PAGE and transferred to nitrocellulose membranes. Membranes were blocked and then probed with the anti-TRPV1 and anti-cytochrome-C antibody. After washing, a horseradish peroxidaseconjugated secondary antibody was used, and the immunoreactive bands were visualized by a chemiluminescence substrate using an Intelligent Dark Box. Optical density was analysed by the Image Pro Plus software.

RNA interference (RNAi)

SZ95 sebocytes were transfected at 50–70% confluence with 40 nM human TRPV1-specific double-stranded small interfering RNA (siRNA) Stealth RNAi oligonucleotides using Lipofectamine 2000 transfection reagent. For controls, RNAi Negative Control Duplexes (scrambled RNAi) were employed, wich does not target any known mRNA sequences. The efficacy of gene scilencing after the transfection was daily evaluated by Q-PCR and western blotting. Experiments were carried out at minimal expression of TRPV1.

Determination of intracellular lipids

For semiquantitative detection of sebaceous lipids, cells were cultured on glass coverslips. Cells were fixed in paraformaldehyde, and lipid droplets were stained with Oil Red O dye. Nuclei were counterstained with Mayer's hematoxylin. Following the colouration, cells were mounted and investigated by microscopy.

For quantitative measurement of lipid content, sebocytes were cultured in 96-well black-well/clear-bottom plates. Subsequently, supernatants were

discarded, cells were washed and Nile Red solution in PBS was added to each well. Fluorescence of Nile Red solved in cellular lipids was measured on a fluorescent microplate reader (FLIPR). 485nm excitation and 565nm emission wavelengths were used to detect neutral lipids, and 540nm excitation and 620nm emission wavelengths for polar lipids.

Ca^{2+}-imaging

Dendritic cells were seeded and cultured on sterile coverslips and a calcium-sensitive probe was introduced into the intracellular space by incubating the cells with acetoxymethil ester-conjugated fura-2 (fura-2 AM) dye. Cells were investigated by a fluorescence microscope. Excitation was altered between 340 and 380 nm using a dual wavelength monochromator, and emission was monitored at 510 nm with a photomultiplier (PTI Deltascan). Capsaicin solved in Tyrode solution were applied through a rapid perfusion system positioned in close proximity to the cell measured. $[Ca2+]_{IC}$ levels were characterized by the ratio (F_{340}/F_{380}) of the fluorescence intensities measured with excitation wavelengths of 340 (F_{340}) and 380 nm (F_{380}).

Determination of viable cell numbers

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

Determination of mitochondrial membrane potential

A decrease in the mitochondrial membrane potential is one of the earliest markers of apoptosis. Mitochondrial membrane potential of cells was determined using a MitoProbe $DiIC_1(5)$ Assay Kit. Cells were cultured in 96-well black-well/clearbottom plates. The carbonyl cyanide 3-chlorophenylhydrazone (CCCP) was used as positive control to decrease mitochondrial membrane potential. After removal of supernatants, cells were incubated with $DilC_1(5)$ working solution, washed, and the fluorescence of $DilC_1(5)$ was measured at 630 nm excitation and 670 nm emission wavelengths.

Determination of cytotoxicity (necrosis)

Necrotic effect of acute (24 h) capsaicin treatment was determined by measuring the glucose-6-phosphate dehydrogenase (G6PD) release. The enzyme activity was detected by a two-step enzymatic process that leads to the reduction of resazurin into orange-fluorescent resorufin. The fluorescence emission of resorufin was monitored at 545 excitation and 590 emission wavelengths.

The cytotoxic effect of long-term capsaicin treatment was determined by SYTOX Green staining. The dye is able to penetrate (and then bind to the nucleic acids) only to necrotic cells with ruptured plasma membranes, whereas healthy cells with intact surface membranes show negligible green fluorescence. Following SYTOX Green staining, fluorescence was measured at 490 nm excitation and 520 nm emission wavelengths.

Determination of phagocytotic activity

Dendritic cells were cultured in 96-well black wall/clear bottom plates. Following the corresponding treatment, cells were incubated by FITC-conjugated *Escherichia coli* bioparticles for 2 hours. Supernatant was removed, cells were washed by trypan blue solution, and the fluorescence intensity of

phygocyted bacteria was measured at 490 nm excitation and 520 nm emission wavelength.

Determination of cytokine release

To determine released cytokines, supernatants were collected, and the released amounts of IL-1β, IL-6, IL-8, IL-10, IL-12 and TNFα were determined using specific OptEIA kits.

RESULTS

TRPV1 is expressed on human sebaceous glands in situ and on human SZ95 sebocytes in vitro

Using immunohistochemistry, we have shown that human sebaceous glands express TRPV1 *in situ*. TRPV1-specific immunosignals were more prominent on the centrally located, more differentiated (matured) sebocytes, than periferal, undifferentiated ones.

Human sebaceous gland derived SZ95 cells also express TRPV1 on the gene and protein level. The TRPV1-specific immunoreactivity was inhomogeneous in the cell culture, possibly because the proliferation and differentiation status of the cells affect the actual expression level of TRPV1. Using western blot and Q-PCR, we have found, that the level of TRPV1-specific mRNA transcripts and of TRPV1 protein markedly increased in parallel with the confluence of cultures, which is proportional to the differentiation status of cultures.

Capsaicin selectively inhibits basal and arachidonic acid-induced lipid synthesis of SZ95 sebocytes, but does not influences the viability

We investigated the effect of TRPV1 agonist capsaicin on the lipid synthesis and therminal differentiation of SZ95 sebocytes. Flow cytometry analysis showed that 48-hour-long application of 10 μM capsaicin was not accompanied by changes in sebocyte size or granulation. Nile red staining-based quantitative fluorometric measurement revealed that capsaicin treatment (up to 24 hours) significantly inhibited basal synthesis of both neutral and polar lipids in a dose-dependent fashion.

Capsaicin did not influence the viabilty of SZ95 sebocytes. 48-hour-long application of 10 μM capsaicin did not altere the percentage of both apoptotic (Annexin-V+/propidium iodide–) and necrotic (propidium iodide+) cells in sebocyte cultures. Capsaicin applied for 24 hrs in a wide concentration range (from 0.1 nM up to 10 μM) did not alter the viable cell number, and did not induce cell death of any form (apoptosis, necrosis), namely, did not decrease the mitochondrial membrane potential (apoptosis) and did not induce G6PD release (necrosis).

Arachidonic acid was reported earlier as an effective inducer of terminal differentiation in SZ95. Therefore, we used arachidonic acid as positive control. The arachidonic acid, also in our experiments, induced the terminal differentiation of sebocytes, it increased both the size and granulation of cells in 48 hours. Using Oil Red O and Nile Red stainings, we have shown that 50 μM arachidonic acid applied for 24 hrs promote the synthesis of neutral lipids, a hallmark of differentiation. The same treatment induced also apoptotic (decrease of mitichondrial membrane potential), but not necrotic (G6PD release) processes. Following a higher dose (100 μM) and longer (48 hrs) arachidonic acid treatment, we observed also late apoptotic/necrotic processes, namely it increased the percentage of Annexin-V+/propidium iodide+ cells.

Capsaicin, similary to basal lipid synthesis, inhibited also the arachidonic acid-induced lipid synthesis, but it was unable to prevent the apoptosis-inducing action of arachidonic acid.

The effect of capsaicin to inhibit lipid synthesis is mediated by TRPV1

The TRPV1 mostly functions as a Ca^{2+}-permeable channel. Therefore, we next investigated the role of Ca^{2+} in the effects of capsaicin. The activity of the capsaicin to suppress basal and arachidonic acid-induced lipid accumulation was abrogated by lowering the extracellular Ca concentration of the culturing medium. These findings proposed that the actions of capsaicin are mediated by TRPV1-specific $[Ca^{2+}]_{IC}$ elevations.

To further investigate the issue of specificity, we used pharmacological methods. The specific TRPV1 antagonist, iodoresiniferatoxin (I-RTX) abrogated the effect of capsaicin to inhibit basal and arachidonic acid-induced lipid accumulation. Intriguingly, we also found that I-RTX moderately augmented the stimulatory effect of arachidonic acid on lipid synthesis. This finding suggests that TRPV1 may have an endogenous activity to inhibit lipid formation.

The TRPV1 specificity of the effect of capsaicin was further assessed by RNA interference (RNAi). SZ95 sebocytes were transfected with siRNA oligonucleotides against TRPV1 mRNA. Western blot and Q-PCR analysis revealed that the expression of TRPV1 was significantly ‘‘knocked-down’’ by both RNAi probes. At day 2 after transfection, when the scilencing was the most effective, treatments with capsaicin and/or arachidonic acid were repeated, then lipidsynthesis was determined. RNAi-mediated knockdown of TRPV1 resulted in the loss of effect of capsaicin to inhibit basal and arachidonic acid-evoked lipid synthesis.

RNAi-mediated silencing of TRPV1 moderately augmented the stimulatory effect of arachidonic acid on lipid synthesis, which also suggest that TRPV1

may have an endogenous constiutive activity to inhibit lipid formation. Collectively, these findings suggest that the action of capsaicin to supress lipid synthesis was specifically mediated by TRPV1.

Capsaicin alters expressions of genes involved in the regulation of lipid synthesis and differentially regulate the release of selected cytokines

Capsaicin treatment was reported to be able to alter the gene expression profile and the cytokine production of treated cells. Therefore, using by Q-PCR, we investigated the gene expression of members of the PPAR and RXR nuclear transcription factor families, which are involved in the regulation of lipid homeostatsis. After 24 hours treatment (that is, the time-point at which lipidsynthesis was strongly suppressed), 1 μM capsaicin significantly reduced the expression of all genes compared to control. These findings suggest that TRPV1-mediated-signaling pathway(s) inhibit the expression of key genes of lipid synthesis.

Amounts of the released IL-1β, IL-6, and TNFα (which proinflammatory cytokines were earlier recognized as product of sebocytes) were determined using specific ELISA technique. Capsaicin treatment selectively decreased the level of IL-1β without affecting that of IL-6 and TNFα.

Prolonged application of capsaicin induces a dose-dependently biphasic alteration in cellular proliferation of SZ95 sebocytes

Previously, we had found that capsaicin, when applied for 3–5 days, markedly inhibits proliferation and induces apoptotic processes of human keratinocytes and hair follicles. Therefore, we also investigated the effect of long-term capsaicin application on the proliferation of SZ95 sebocytes.

For this experiment, the sebocytes were seeded in high density, and the serum content of the culture medium was decreased to 3%. Under these culture

conditions, the SZ95 sebocytes fully survived but exhibited only an insignificant growth rate. We have found, that low doses (0.01–100 nM) of capsaicin stimulated the proliferation, but higher doses (>1 μM) of capsaicin reduced the number of viable cells. Suppression of the $[Ca^{2+}]_{EC}$ or prescence of I-RTX completely prevented the growth-promoting action of low capsaicin concentrations, which findings suggest, that effects the growth-promoting effect of low capsaicin doses was indeed mediated by TRPV1. In contrast, decreased $[Ca^{2+}]_{EC}$ or I-RTX did not modify the growth-inhibitory and cytotoxic effect of high concentrations of capsaicin, suggesting a receptor independent effect of the high-dose capsaicin.

Following a 6-day-long treatment, higher doses (1–30 mM) of capsaicin damaged the integrity of cellular membranes and induced necrotic cell death (indicated by SYTOX Green staining).

Human monocyte-derived dendritic cells express TRPV1

First, we measured the existence of TRPV1 on monocyte-derived dendritic cells. Monocytes isolated from human periferial blood were differentiated to immature dendritic cells using IL-4 and GM-CSF for 5 days. During differentiation, the monocytes specific CD14 dissapeared and the expression of dendritic cell specific DC-SIGN was induced.

Both human peripheral blood monocytes as well immature dendritic cells express TRPV1 at the gene and protein levels. We also found that TRPV1 expression dramatically increased during the cytokine-induced differentiation of immature dendritic cells. The increase in protein expression followed the increase of mRNA level with a day delay, wich can based on an increased turn over of the receptor during differentiation. This finding suggested the potential role of TRPV1 in differentiation process.

The TRPV1 expressed on dendritic cells was found to work as functional Ca^{2+}-permeable channel. Acute administration of capsaicin elevated the $[Ca^{2+}]_{IC}$

of both immature and mature dendritic cells. This effect was reversible, terminating the capsaicin administration the $[Ca^{2+}]_{IC}$ was reverted. The reactivity of dendritic cells was controled by using ATP as positive control.

Activation of TRPV1 inhibits differentiation of human monocyte-derived dendritic cells

We then investigated the effect of the activation of TRPV1 on the differentiation of dendritic cells. Cells were treated with capsaicin and/or with combination of IL-4 and GM-CSF, then the expression of DC-SIGN was monitored. Long-term application (for 5 days from day 0) of capsaicin (without IL-4 and GM-CSF) did not induce the in vitro differentiation of monocytes to immature dendritic cells (DC-SIGN expression was not induced). As metioned above, the IL-4 and GM-CSF evoked the DC-SIGN expression, and induced the differentiation of mononcytes to dendritic cells. However, if the above treatments were combined, capsaicin applied daily from day 0 markedly lowered the DC-SIGN expression, indicating inhibited cytokine-induced differentiation of dendritic cells. Capsaicin also reduced the expression of CD11c and HLA-DR, other markers of dendritic cells.

At day 5, we have also investigated the effect of capsaicin on the viability of the cells. Capsaicin applied daily from day 0 did not significantly alter the viable cell number and it did not induce cell death of any form: it induced neither apoptosis nor necrosis indicated by mitochondrial membrane potential and SYTOX Green staining, respectively.

Then, we investigated the role of TRPV1 in mediating the effect of capsaicin. The above capsaicin treatments were repeated in the prescence of TRPV1 specific antagonist capsazepine (applied also daily form day 0). The capsaicin inhibited the DC-SIGN expression in a dose-dependent fashion. The capsazepine effectively prevented the action of capsaicin to suppress DC-SIGN

expression. These findings suggested, that the effect of capsaicin on differentiation of dendritic cells was most probably mediated by TRPV1.

When applied from day 3, the TRPV1 agonist was ineffective in modifying the differentiation of immature dendritic cells. This latter finding suggests that the capsaicin interferes with the early events of dendritic cell differentiation.

Activation of TRPV1 inhibits the phagocytosis of human monocytes derived immature dendritic cells

Phagocytosis of immature dendritic cells (differentiated by IL-4 and GM-CSF) was assessed by internalization of FITC-conjugated *Escherichia coli* bioparticles. In those cells, which were treated by capsaicin from day 0 for 5 days, the phagocytotic activity was significantly suppressed. In addition, we also found that the co-application of capsazepine prevented the action of prolonged capsaicin administration again arguing for the TRPV1-specificity of the effect. of capsaicin. Likewise expression of differentiation markers, the functional activity (phagocytosis) of immatured dendritic cells was also inhibited by capsaicin in a TRPV1 specific manner.

We then measured the effect of ‘‘acute” application of capsaicin on the phagocytosis of the differentiated immature dendritic cells. Following the IL-4 and GM-CSF induced differentiation, immature dendritic cells were treated by capsaicin only at day 5, then internalization of FITC-conjugated *E. coli* bioparticles was measured. As short as 2-hour-long capsaicin treatment significantly inhibited the phagocytotic activity, which antagonized by capsazepine suggesting a TRPV1 specific action of capsaicin. The suppression of extracellular Ca^{2+}-concentration also abrogated the effect of capsaicin to inhibit phagocytosis, again suggesting that TRPV1 indeed functions as a Ca^{2+}-permeable channel on immature dendritic cells.

Capsaicin did not induce the maturation of humanmonocite-derived immature dendritic cells, but inhibits activation and maturation induced by proinflammatory cytokines

We also assessed the role of TRPV1 in the maturation and activation of immature dendritic cells. Capsaicin treatment for 24 hrs failed to induce the maturation of immature dendritic cells as measured by assessing the expression of the maturation marker CD83 and migration marker CCR7.

In contrast with capsaicin, the proinflammatory cytokine cocktail induced the expression of both CD83 and CCR7 indicating the maturation and activation of deendritic cells. Coapplication of capsaicin inhibited the maturation induced by the proinflammatory cytokine cocktail, namely it suppressed the expression of CD83 and CCR7. Likewise, capsaicin also decreased the expression of HLA-DR as well as other co-stimulatory molecules (CD40, CD80, and CD86) induced by the pro-inflammatory cocktail.

The proinflammatory cytokin cocktail also elevated the mRNA expression of some cytokines (IL-6, IL-8, IL-10, and IL-12) in dendritic cells, but did not altered the expression of others (TNFα, IL-1β). Coapplication of capsaicin selectively inhibited the production of IL-6 and IL-12 transcripts, but did not affect the highly elevated synthesis of the IL-8. Intriguingly, capsaicin further elevated the expression of the rather anti-inflammatory IL-10.

Using ELISA technique, we also determined the released amount of IL-6, IL-8, IL-10 and IL-12. Likewise the mRNA expression, the release of IL-6 and IL-12 from mature dendritic cells was also suppresed by capsaicin. Capsaicin also increased the IL-10 release and did not affect the release of IL-8. The prescence of capsazepine also abrogated the effect of capsaicin on cytokine release, suggesting that the effect of capsaicin was also mediated by TRPV1.

DISCUSSION

Activation of TRPV1 blocks the differentiation and lipid synthesis of human sebocytes

In human skin the expression of TRPV1 was shown not only on sensory neurons, but numerous non-neural cell types as well, such as the keratinocytes of the epidermis and hair follcile, sweat glands, the endothelial and smooth muscle cells of veins, mast cells, and the dendritic cells of the sebaceus gland and epidermis, the Langerhans cells. The functionality of the receptor has been verified on both normal human keratinocytes and the HaCatT keratinocyte cell line. Our group has also shown it's function in regulating the biological processes of the hair follicle. In our present work we examined the function of TRPV1 on human sebocytes and monocyte-derived dendritic cells.

We have previously shown the expression of TRPV1 on human sebaceus glands and SZ95 sebocytes. The receptor's expression is inhomogenous and presumably increases during the differentiation of the cells. This is supported by the following: (1) the expression of TRPV1 is stronger on differentiated sebocytes *in situ* and (2) we found higher TRPV1 expression in SZ95 sebocytes with higher confluence *in vitro*. We have previously proven that the expression of TRPV1 increases during the anagen-catagen transition, and that capsaicin treatment promotes the development of the catagen phase.

After a 24 hour capsaicin treatment the sebocytes did not show any sign of terminal differentiation; neither lipid synthesis nor apoptosis was found to be increased. We did not experience any change in the morphology or viability of the cells, however capsaicin decreased the sebocytes' basal lipid synthesis.

The terminal differentiation of sebocytes could be induced with arachidonic acid, which increased both the cells' lipid synthesis and induced apoptosis. Capsaicin selectively blocked the increase of lipid synthesis caused by

arachidonic acid, but did not effect the induction of apoptosis. The effect of capsaicin could be suspended with both pharmacological (I-RTX) and biological (RNAi) pertrubation of the receptor.

TRPV1-specific effects were described on normal human keratinocytes, the HaCaT cell line and the outer root sheath (ORS) keratinocytes, where capsaicin induced the cells' differentiation, apoptosis, IL-8 and PGE_2 production through a TRPV1-Ca^{2+} mediated pathway. In our present experiments decreasing the extracellular Ca^{2+} concentration blocked the effects of capsaicin, which further supports the role of TRPV1 as a Ca^{2+} channel in this experimental setup.

TRPV1 mediated $[Ca^{2+}]_{IC}$ increase has been shown to block proliferation and induce apoptosis on glioma cells, thymocytes and keratinocytes. In contrast capsaicin applied for 24 hours had no effect on the viability of the cells, did not induce either apoptosis nor necrosis and did not influence apoptosis caused by arachidonic acid. Indeed, long-term low-dose capsaicin treatment TRPV1 specifically heightened the proliferation of SZ95 sebocytes. Based on the above TRPV1 activation shows highly varied effect on different cell types even in the same organ, for example keratinocytes and sebocytes in skin. One explanation for this phenomenon is that changes in Ca^{2+} concentration effect the function of the cells in different ways. High Ca^{2+} concentrations cause differentiation in keratinocytes, while a similiar effect can be evoked in sebocytes with low extracellular Ca^{2+}, and high concentrations increase the proliferative capacity of the cells, while inhibiting their differentiation.

Contrary to capsaicin arachidonic acid increased the differentiation of SZ95 sebocytes and their lipid synthesis while inducing apoptosis. Although derivatives of arachidonic acid (eicasonoids, endocannabinoids) can activate TRPV1 it appears that the receptor plays no part in the mediation of this effect. In other experiments we have found that endocannabinoids increase the differentiation of SZ95 sebocytes through the CB2 cannabinoid receptor.

Arachidonic acid and its lipoxygenase products as well as endocannabinoids can act as activators of PPARs. PPARs are important mediators of lipid metabolism and as such they play important roles in the regulation of the sebocyte's lipid production. In our experiments capsaicin decreased the expression of both PPARs and their partners in heterodimers, RXRs.

Sebocytes are capable of producing inflammatory mediators (IL-1β, IL-6, IL-8/CXCL8, TNFα, LTB_4, PGE_2) which can be increased by inflammation inductors. They can play a central role in the development of inflammatory syndromes such as acne.The developement of acne requires multiple pathological processes such as the hyperproliferation of keratinocytes, increased lipid synthesis of sebocytes, where the composition of the lipid product is also changed and the proliferation of pathogenic microorganisms.. The production of inflammatory mediators can be increased during the process, which causes leukocytes to infiltrate the affected follicles. Acne can develop without pathogenic microorganisms, if the production of inflammatory mediators is increased because of PPAR activation or increased androgen effect. In *in vitro* experiments arachindonic acid can cause changes in sebocytes that mimic acne, such as increasing the production of lipids and inflammatory medirators. Since the activation of TRPV1 inibited the increased lipid synthesis, expression of PPAR genes and release of pro-inflammatory IL-1β caused by arachindonic acid, this raises the possibility of using the pharmacological activation of TRPV1 in the therapy of acne.

TRPV1 inhibits the differentiation, maturation and activation of human dendritic cells

Similiarly to Langerhans cells both immature and mature dendritic cells differentiated from monocytes seperated from peripherial blood express

functional TRPV1. The expression of TRPV1 increases drastically during differentiation of monocytes into dendritic cells, which raises the possibile role of the receptor in the process.

Capsaicin applied daily did not induce the differentiation of monocytes into dendritic cells, however it did inhibit the differentiation caused by IL-4 and GM-CSF, as measured by the expression of dendritic cell markers DC-SIGN and CD11c, as well as the expression of HLA-DR. The constatnt presence of capsaicin also inhibited the phagocytotic activity of the cells, without interfering with their viability. This effect of capsaicin was presumably relayed through TRPV1, since it could be antagonized with capsazepine.

Human monocyte-derived dendritic cells treated with capsaicin for 24 hours did not cause the maturation of the cells, but even a two hour long capsaicin treatment decreased the cells' phagocytosis in a TRPV1 specific, Ca^{2+} mediated manner. Accordingly the activation of TRPV1 inhibits not only the differentiation of monocytes into dendritic cells, but also their function once differentiated.

Immature dendritic cells induced with a combination of cytokines showed increased expression of the migratory marker CCR7 and also HLA-DR along with many costimulatory molecules (CD40, CD80, CD83, CD86) and increased transcription of certain cytokines (IL-6, IL-8, IL-10, IL-12). Capsaicin applied during the induction described above TRPV1 specifically inhibited the increase of migratory and maturation markers along with the production of proinflammatory IL-6 and IL-12, while increasing that of the antiinflamatory IL-10.

Monocyte-derived dendritic cells are considered good models of myeloid dendritic cells, and it has proven in mouse models that Langerhans cells can differentiate from monocytes *in vivo*. During the monocyte-dendritic cell transformation – similairly to the regulation of sebocytes – PPARγ plays an important role, which is marked by its increased activity and expression. In

dendritic cells activated with LPS the activity of PPARγ decreases the expression of certain maturational markers and also inhibits their citokine production.

Capsaicin, similairly to PPAR ligands, also inhibited the differentiation of dendritic cells and decreased the expression of maturational markers and the production of certain interleukins. Activation of TRPV1 blocked the early events of monocyte-dendritic cell transformation, when the activation of PPARγ is the most pronounced. It is therefore imaginable that – similairly to sebocytes – PPARγ plays a role in the capsaicin induced TRPV1 signalization. According to the latest research TRPV1 signalization can block the expression and function of PPARγ: activation of TRPV1 expressed on adipocytes ellicited an influx of Ca^{2+} which decreased the expression of PPARγ and blocked their differentiation and triglyceride synthesis.

On human monocyte-derived dendritic cells both capsaicin and ATP (which was used as a positive control) increased the $[Ca^{2+}]_{IC}$. The presence of numerous – both ionotrope and metabotrope – purinoreceptors was previously proven on these cells, where the application of ATP was found to increase the $[Ca^{2+}]_{IC}$ and also their maturation. According to other reports ATP acting through ionotropic receptors can inhibit the production of certain proinflammatory citokines and the developement of T_h1 immune response. All these data suggest that Ca^{2+} coupled signalization can have a versatile influence on the function of dendritic cells depending on the receptor activated, the character of Ca^{2+} concentration changes or the presence of other stimulatory signals.

Increasing the level of cyclic nucleotides blocks the formation of CD1a+ and CD14- dendritic cells as well as their subsequent LPS induced maturation, but has no effect on DC-SIGN while increasing the expression of MHC-II molecules. In other words cAMP/cGMP – unlike TRPV1 mediated processes – does not inhibit the early steps of monocyte-dendritic cell transformation.

According to our results the activation of TRPV1 on monocyte-derived dendritic cells has an antiinflammatory effect since it negatively effects their differentiation (DC-SIGN expression), phagocytosis, antigen presentation (HLA-DR expression), migration (CCR7 expression), induced maturation (the expression of co-stimulatory molecules) and the production of certain proinflammatory citokines (IL-6, IL-12), while increasing the production and release of IL-10.

The antiinflammatory effect of the TRPV1 activator capsaicin has been reported on in numerous other works, and this effect can be transmitted through several mechanisms. Capsaicin can (1) through constant activation of TRPV1 expressing sensory neurons decrease the amount of neuropeptides responsible for neurogenic inflammation or increase the release of antiinflammatory mediators (somatostatin); (2) block the function of macrophages through the NF-κB pathway and inducible nitrogen-monoxide synthase as well as the inhibition of cyclooxygenase; (3) according to our present experiments through TRPV1 block the differentiation and maturation of dendritic cells.

In contrast to our results in murine dendritic cells derived from bone marrow the activation of TRPV1 was found to increase their maturation and activation and cause subsequent proinflammatory signalization. The difference in results can be explained by differences in species (mouse vs human), in culture conditions (bone marrow vs monocyte derived dendritic cells) or in the experimental setup (partly *in vivo*, KO mice vs *in vitro*). During *in vivo* application TRPV1 activation can cause neuropeptide release from sensory neurons which can also effect the activation of the cells. In our present work we used *in vitro* application where such factors are not present.

Other experiments on murine bone marrow-derived dendritic cells could not prove the expression of TRPV1, but did find the closely related TRPV2. Previous works have shown the possibility of TRPV1 and TRPV2

heteromerization, but currently we have no such data available on dendritic cells.

TRPV1 plays a versatile role in the regulation of biological processes in skin

In our present work we have described the functional expression of TRPV1 on human sebocytes and dendritic cells, thereby further expanding our knowledge of the role of TRPV1 in the regulation of the biological processes of skin.

TRPV1 is expressed in skin on numerous cell types, where its activation causes varied effects. The activation of the receptor on some cells can also influence the function of other cells by, for example, the release of mediators. TRPV1 expressed on sensory neurons plays a role in the transmission of painful stimuli and thermal hyperalgesia, and as such the desensitisation of neurons through TRPV1 can be exploited in the therapy of pain syndromes. The activation of TRPV1 can also lead to neuropeptide release and subsequent neurogenic inflammtion. Certain neuropeptides can inhibit the immune function of dendritic cells in skin, but SP is mainly proinflammatory and also causes the terminal differentiation of sebocytes besides activating immune cells which can theoretically contribute to the developement of acne. In this case TRPV1 expressed on neural elements (proinflammatory) and sebocytes (antiinflammatory) can transmit opposing effects.

A limited subset of TRPV1 expressing neurons also transmit pruritogenic stimuli, thus the activation/desensitization of neuronal TRPV1 may influence the developement of itch. TRPV1 can also cause the release of pruritogen mediators from non-neuronal elements of skin, but it can also contribute to the beneficial effect of capsaicin in diseases linked with pruritus through desensitization. Some lipid mediators capable of activating TRPV1 (for example anandamid) can also activate cannabinoid receptors, which also appears to have antipruritogenic effects. Anandamide can also be released after TRPV1 activation, it is likely that

there is a functional interaction of the endovanilloid and enocannabinoid systems in the modulation of itch.

According to our results TRPV1 expressed on keratinocytes, hair follicles and sebocytes can transmit opposing signals in the biological processes of skin. In keratinocytes and hair follicles the activation of the receptor causes differentiation and apoptosis while decreasing proliferation and inducing catagen transformation, while on sebocytes it decreases both the cells' differentiation and lipid synthesis while subsequently increasing their proliferation. On keratinocytes it increases the production of proinflammatory mediators while it decreases the IL-1β release in sebocytes. In this way the activation of TRPV1 on keratinocytes and sebocytes can influence the sensory and immunological processes of skin in a contradictory fashion. The question is further nuanced by the fact that these cells can produce numerous mediators (PGs, LTs, ILs, etc.) which can influence the function of TRPV1 in an autocrine and/or paracrine fashion.

The immunological effect of TRPV1 can also be relayed through the immune cells (e.g.: mast cells and Langerhans cells) of skin. Capsaicin causes an influx of calcium and IL-4 release on mast cells, and in our present experiments we have shown the functionality of TRPV1 on human dendritic cells.

The myeloid dendritic cells of skin are heterogenous, with Langerhans cells and dermal dendritic cells constituting the two major populations. Dermal dendritic cells can be further characterized, with the distinction between resident and inflammatory DCs being most important. These subsets differ in their functional characteristics as well, with Langerhans cells having a role in the developement of immunotolerance, while the number of inflammatory DCs is increased in certain inflammatory conditions where they play an important role in the given syndrome.

The use of pharmacons to influence TRPV1 on dendritics cells can presumably be used in numerous dermatological/immunological syndromes. According to our *in vitro* studies the activation of TRPV1 expressed on monocyte-derived dendritic cells has an antiinflammatory effect. On the other hand we have no information regarding the sensitivity of other cell types to TRPV1 activation, nor what role they might in the antiinflammatory effect of capsaicin. Because of this many more experiments are required to further ellucidate the role of TRPV1 expressed on dendritic cells in the complex regulation of the immune functions of skin.

SUMMARY

The aim of the current study was to investigate the role of TRPV1 in biology of human sebaceous gland derived SZ95 sebocytes and monocyte-derived dendritic cells *in vitro*. Like the human sebaceous glands, SZ95 sebocytes also expressed TRPV1. The TRPV1 activator capsaicin inhibited both the basal and arachidonic acid induced lipid synthesis (but did not influence the viability of the cells). The specific role of TRPV1 in mediating these effects was evidenced by using both the specific antagonist I-RTX and the RNAi technique. Results of the experiments carried out in decreased Ca^{2+} containing medium suggested that the TRPV1 operate as a Ca^{2+} channel. Activation of TRPV1 altered the expression of transcripition factors regulating lipid synthesis as well as the production of selected cytokines. Following long-term treatment, low concentrations of capsaicin increased the proliferation rate of SZ95 sebocytes acting via TRPV1, whereas high doses decreased the viability of the cells independently of TRPV1. Functional TRPV1 channel was also identified on human monocytes and monocyte-derived dendritic cells. Dendritic differentiation was inhibited by capsaicin in a TRPV1-dependent manner: capsaicin supressed both the expression of dendritic differentiation markers and the phagocytosis of the cells. Moreover, stimulation of TRPV1 on differentiated, immatured dendritic cells did not induce the maturation, but decreased the phagocytosis and inhibited the maturation induced by pro-inflammatory cytokines: capsaicin suppressed the expression of maturation markers and the production of proinflammatory cytokines. In parallel, the production of the anti-inflammatry cytokine IL-10 was increased by capsaicin. Our results suggest that activation of TRPV1 suppresses the lipid synthesis/differentiation of sebocytes and have an anti-inflammatory effect on dendritic cells. These data argue for the potential role of TRPV1 to influence immune processes of the human skin as well as of certain dermatoses with altered lipid homeostasis (e.g. acne vulgaris).

PUBLICATIONS

The thesis was built on the following in extenso publications:

Tóth BI, Géczy T, Griger Z, Dózsa A, Seltmann H, Kovács L, Nagy L, Zouboulis C C, Paus R, Bíró T. (2009): Transient receptor potential vanilloid-1 signaling as a regulator of human sebocyte biology. *J Invest Dermatol. 129(2)*, 329-339. **IF:5,251***

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