SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

The role of the transient receptor potential vanilloid (TRPV)-3 ion channel in the regulation of inflammatory processes in human epidermal keratinocytes

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UNIVERSITY OF DEBRECEN
DOCTORAL SCHOOL OF MOLECULAR MEDICINE

DEBRECEN, 2022

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The Examination takes place at Department of Physiology Faculty of Medicine, University of Debrecen
Debrecen, October 29, 2020, 14 pm

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Debrecen, December 02, 2022, 13 pm

INTRODUCTION

In today's world, great emphasis is placed on our aesthetic appearance, and especially on regular skin care, which is part of our daily routine. To cater to this need, the beauty industry offers users an inexhaustible supply of cosmetics. Ingredients in cosmetics "promise" a number of benefits for more beautiful, healthier skin. However, in order for a skin care agent to be marketed and used safely, numerous clinical and molecular biological research results are required. Nowadays, it is possible to study different active substances not only at the tissue but also the cellular level – by elucidating which receptor(s) they act on, how they affect the normal function of cells, and through which signaling pathway they act. In addition, the mapping of cognate receptors on the cells themselves and their role in biological processes can be studied by a host of molecular methods. Numerous research groups around the world are working to gain an accurate understanding of the biology of the skin, as it is our "organ" that is directly exposed to environmental damage. At the same time this provides an added benefit, as in the case of skin diseases, the therapeutic options can be applied not only systemically, but also topically, directly to the affected skin area. Our laboratory - partly in collaboration with cosmeceutical companies - has been involved in this research for a number of years.

The main profile of our research group is the study of the endocannabinoid system (ECS), which has greatly expanded due to the research of the last decades. Within the ionotropic receptors that are part of the ECS, several members of the vanilloid

subfamily of transient receptor potential (TRP) channels have been identified in components of the skin (hair follicles, pilosebaceus unit, keratinocytes).

Research in recent years has shown that a mutation that causes hairless phenotypes and skin diseases in certain rodents is found in the region encoding the *Trpv3* gene. Interestingly in patients with Olmsted's syndrome with similar phenotypes and symptoms, mutations affecting the same genetic region can be detected, as well as several other mutations of the same gene. In our present work, we examined the transient receptor potential vanilloid-3 (TRPV3) ion channel on human epidermal keratinocytes which form the outermost layer of the skin. We performed our experiments on healthy tissues and keratinocytes isolated from them, as well as samples from patients with atopic dermatitis (AD), one of the most common skin diseases. The aim of our research was to investigate the extent to which the TRPV3 ion channel is expressed in healthy and AD keratinocytes, whether the channels are present in a functionally active form, and to determine if the activated channel plays a role in the development and maintenance of inflammatory processes in the skin. If the channel is actively involved in these biological processes, activation or inhibition of the TRPV3 ion channel may be an effective therapeutic target in the treatment of inflammatory skin diseases.

BACKGROUND

Transient receptor potential vanilloid (TRPV)-3 ion channel

The transient receptor potential vanilloid-3 (TRPV3) ion channel is perhaps one of the most special members of the vanilloid subfamily. It shows approximately 43% sequence homology with TRPV1, which is the first member of the subfamily to be described. The thermosensitive ion channel - which is activated in the physiological heat range (<33 ° C) -, forms a homo- or heterotetrameric form, similar to other TRP channels. Also similarly to other TRP channels TRPV3 subunits contain 6 transmembrane domains. The intracellular amino-terminal end contains 6 ankyrin repeats, creating the "ankyrin repeat domain" (TRPV3-ARD), which differs very little from TRPV1-ARD. Similarly to TRPV1, the conserved region between AR2 and AR3 is involved in the binding of ATP and calmodulin (CaM). In addition, TRPV3 contains a heat-activated, Ca^{2+}-sensitive region and a 2-APB binding site.

TRPV3 is primarily a non-selective cation channel; during activation the permeable pore of the channel dilates, and the negatively charged amino acids in the pore facilitate the passage of larger cations.

Of the TRPV channels, TRPV3 is the only one in which no endogenous modulator or ligand has been described, but several exogenous, including synthetic and naturally occurring, primarily aromatic compounds are activators of the channel. Among the synthetic compounds, the best known activators are 2-APB and drofenine. Its natural activators include a number of aromatic compounds that also create a pleasant warm

feeling, thereby potentiating the response of TRPV3 to temperature, such as camphor, carvacrol, thymol, and eugenol.

In contrast to other TRP channels, TRPV3 shows low expression on sensory neurons. It is expressed in several organs, such as the brain, tongue, testes, cornea, distal colon, larynx, and inner ear, but has one of the highest expression profiles in the skin, primarily in epidermal keratinocytes and hair follicle cells.

The primary defense system of our skin is the epidermal barrier

One of the most important functions of human skin is to form a barrier, which is a primary system of protection against the harmful effects of the external environment. The barrier system of our skin is created by a very complex ensemble of several functions, in the formation of which the dermal and epidermal layers of the skin participate in coordination with each other. Within this system the keratinocytes that make up the epidermis play a key role, primarily in the *stratum corneum*.

The main members of this complex system, which requires coordinated regulation, are the *biological barrier* that creates and regulates the commensal flora; a *chemical barrier* that ensures skin hydration, fluid balance, and proper pH; a *physical or mechanical barrier* that provides skin strength and is resistant to mechanical effects; and an *immunological barrier* that responds to the entry of pathogenic microbes.

“Prototype” of diseases affecting the balance of the epidermal barrier: atopic dermatitis

Atopic dermatitis (AD) is one of the most common chronic inflammatory skin diseases; in terms of its symptoms, it is most characterized by recurrent eczema, dry skin, and severe itching, resulting in a deterioration in the quality of life for millions of people worldwide. It is very often seen as a “prototype” of barrier diseases, as all participants in the epidermal barrier are disturbed, and inflammation of the skin and severe itching also contribute greatly to the development, progression, and chronicity of the disease. It should be emphasized that inflammation, barrier dysfunction, and itching are interrelated, mutually reinforcing processes that are jointly responsible for the development of the disease.

The connection between the TRPV3 ion channel and the skin

Although the presence of TRP ion channels was first described on neurons, research in recent decades has shown that many TRP channels are also expressed on non-neuronal cell types. New findings have highlighted the role of TRP channels in the skin, where they have been shown to play an important role in a number of processes in skin cells. One of the most interesting in this respect is the TRPV3 ion channel, which was first described on keratinocytes.

The significance of TRPV3 expression in the skin is confirmed by the fact that the hairless phenotype of the DS-Nh mouse strain in keratinocytes is caused by a single point mutation in the region encoding the TRPV3 gene, namely the Gly573Ser point mutation leading to a “gain-of-function” mutation. The affected mice are characterized not only

by a hairless phenotype but also by a number of skin lesions such as spontaneous dermatitis, pruritus and hyperkeratosis. Moreover, colonization of Staphylococcus aureus, higher IgE and IL-4 levels, and increased $CD4^+$ T cell infiltration suggest that the TRPV3 ion channel may play a complex role in skin pathophysiology.

The importance of these results is further underlined by the evidence that the same Gly573Ser point mutation as well as several missense mutations in the gene encoding the TRPV3 ion channel lead to the development of a very rare disorder, Olmsted's syndrome. The most common symptoms in the disease include palmoplantar keratoderma, periorophicial hyperkeratosis, diffuse hair loss, baldness and itching. Concomitant histological findings describe hyperkeratosis, parakeratosis, and psoriasis-like lesions with mast cell infiltration in the upper dermis, similar to those described in the aforementioned mice. At the molecular level as well, TRPV3 activation has been shown in keratinocytes to result in the release of signaling molecules (such as prostaglandin E2, ATP, NGF, and TSLP) that cause inflammation and pruritus.

Consistent with these results, our group previously investigated the role of TRPV3 in hair follicles and outer root sheath keratinocytes, where they are expressed in a functionally active form, and their activation inhibits hair growth and cell proliferation and induces cell death.

Importantly for our research, the expression of TRPV3 is increased at the mRNA level in the lesion skin of patients with AD, and the inhibition of TRPV3 by various compounds has been shown to be effective in alleviating AD-like symptoms in animal models. This knowledge has been supplemented by recent findings that TRPV3

expression is also elevated at the protein level in AD lesions, and keratinocytes isolated from the lesion skin of AD patients show increased heat sensitivity in a TRPV3-dependent manner, resulting in the release of pruritogens.

Interestingly, despite previous results, we do not yet have sufficient information on the cellular role and function of TRPV3 in keratinocytes, and previous studies have not examined in detail whether TRPV3 expression and function are altered in non-lesioned skin of AD patients, presumably different from healthy skin. with respect to terminal differentiation and certain immune processes.

AIMS

The effect of TRPV3 ion channel activation on human epidermal keratinocytes is not yet fully elucidated, so we aimed to:

1. Study TRPV3 ion channel expression and functional activity on human epidermal keratinocytes on the molecular level.
2. Investigate the role of TRPV3 in the inflammatory processes of epidermal keratinocytes during activation with classical agonists, and to map signaling pathways involved in these processes.
3. We sought to assess and compare the expression and functional characteristics of the TRPV3 ion channel in samples from patients with AD from non-lesional and lesional areas, as well as on tissues from healthy skin.

MATERIALS AND METHODS

Isolation and culture of epidermal keratinocytes from healthy and AD patients

Healthy skin samples were obtained from dermatologically healthy volunteers who underwent surgery. In atopic individuals, only the upper epidermal layer was removed after proper anesthesia, both from the area of the affected skin lesion and from the region that appeared to be healthy. The experiments were performed after obtaining the permission of the Regional and Institutional Research Ethics Committee of the University of Debrecen (protocol number: DE OEC RKEB / IKEB 3724-2012 case number: IX-R-052 / 01396-2 / 2012) and in compliance with the guidelines of the Declaration of Helsinki.

The samples were cut into 3-5 mm^2 squares. Tissue sections from healthy individuals were digested in 2.4 U / ml dispase overnight and the dermal-epidermal layer was separated using forceps. Both healthy and atopic epidermis were digested with 0.1% trypsin-0.2% EDTA for one hour at 37 °C. Vortexing was performed to mechanically aid in cell separation. DMEM supplemented with 10% w / v fetal bovine serum (FBS) and penicillin streptomycin was used to inactivate trypsin. The cells were centrifuged at 1000 rpm for 8 minutes, and the resulting epidermal keratinocytes were cultured in DMEM / HAM F12 3: 1 supplemented with the specified substances for 2 days. The culture was performed in a humidified air thermostat at 37 °C with 5% CO_2. Following cell adhesion (2-5 days depending on the donor), keratinocytes were cultured in serum-free EpiLife solution supplemented with 5% w / v human keratonicyte growth supplement (HKGS).

The cells were passaged at a confluence level of 80%, thus preventing confluence-induced differentiation of the cells.

Immunohistochemistry

Sections 5 μm thick were made from paraffin-embedded tissue blocks. After deparaffinization and antigen digestion, sections were incubated with primary anti-human TRPV3 antibody overnight at 4 °C. After staining with the primary antibody, sections were incubated with biotinylated goat IgG. Diaminobenzidine (DAB) was used to detect the immune response, and hematoxylin was used for nuclear staining. To verify the labeling procedure, the primary antibody was omitted from the negative controls. Immunostaining was examined and evaluated using a Nikon Eclipse E600 fluorescence microscope.

Immunocytofluorescence

Keratinocytes were plated on glass coverslips and cultured to confluence. After fixation with acetone, the cells were permeabilized and incubated with the primary antibodies at 4 °C overnight. For fluorescent staining, coverslips were incubated with fluorescein isothiocyanate (FITC) -conjugated secondary antibodies for 60 min. 4′,6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei. To verify the labeling procedure, the primary antibody was omitted from the negative controls. Immunostaining was examined and evaluated using an Olympus Xcellence RT fluorescence microscope.

Investigation of gene expression changes

In our experiments, the expression changes of different genes were examined by quantitative “real-time” polymerase chain reaction (RT-qPCR) following reverse

transcription. Total RNA content was isolated using TRIzol reagent following the manufacturer's instructions. Subsequently, starting from 1 μg of total RNA, reverse transcription was performed using the High-Capacity cDNA Reverse Transcription Kit. From the cDNA thus prepared, a specific transcript of the genes was detected by quantitative real-time polymerase chain reaction using TaqMan primers and probes.

TRPV3 protein level detection (Western blot)

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed after determining the protein content of the homogenized samples. The protein was then transferred from the gel to a nitrocellulose membrane. Membranes were incubated with the relevant primary antibodies at 4 °C overnight. Next day, the membranes were incubated with secondary specific IgG antibody for 60 minutes at room temperature. Immune response results were visualized using the SuperSignal West Pico Chemiluminescent Substrate-Enhanced Chemiluminescence Kit, and signals were recorded using the LAS-3000 Intelligent Dark Box and KODAK Gel Logic 1500 Imaging System. ImageJ image analysis software was used to determine the optical density.

Cytokine release assay (ELISA)

To determine the cytokines produced and released by keratinocytes exposed to different treatments, the cell supernatant was collected and the amount of released cytokines (IL-6 and IL-8) was determined using specific ELISA kits following the manufacturer's protocol. At the end of the test, the absorbance was measured at 450 or 405 nm. The amount of cytokine released was determined using a standard curve.

Patch-clamp test

For patch-clamp measurements, keratinocytes (NHEK, AD-NHEK and AD-HEK) were plated on a 10 mm diameter glass cover slip. The coverslips were placed in a perfusion chamber equipped with an inverted microscope and the cells were continuously perfused with Tyrode's external solution. A Multiclamp-700B intracellular amplifier in a whole-cell configuration was used to record the ion currents. The gigaseal (> 1 GΩ) resistance was created by light suction, the whole cell configuration by additional light suction or 1.5 V, 1 ms electrical pulses. The measured ion currents were normalized to the capacity of the given cell, and depolarizing pulses between -10 mV and -20 mV were used to determine this. Electrical signals were recorded at 10 kHz and analyzed using pClamp 10.3 software.

Determination of intracellular calcium concentration ($[Ca^{2+}]_{IC}$)

Keratinocyte cells were plated at a density of 10,000 cells / well in 96-well black-walled, clear-bottomed plates, and the dishes were kept in a thermostat at 37 °C for 24 hours. On the day of measurement, the cells were incubated with 1 μmol/l calcium-sensitive fluorescent Fluo-4 acetoxymethyl ester (dissolved in Fluo-4 AM Hank) at 37 °C for 30 minutes. The Ca^{2+} signal of Fluo-4 AM-loaded cells was measured using a FlexStation II fluorescent microplate reader. At the 30th second of the kinetic measurements, the appropriate concentrations of treatment agents were automatically added to the cells using the pipettor head built into the instrument. The resulting $[Ca^{2+}]_{IC}$ changes were detected at 490 nm excitation and 520 nm emission wavelengths.

Determination of viability (MTT assay)

A colorimetric MTT assay was used to examine cell viability. The method is based on the cleavage of the tertrazolium ring in the yellow methylthiazole tetrazolium (MTT) powder by the mitochondrial dehydrogenase enzyme of living cells, resulting in the formation of purple formazan crystals, the amount of which can be measured colorimetrically after their acidic dissolution. Formazan crystals formed in cells treated with different concentrations of the corresponding pharmacons were dissolved in "MTT solubilizing solution" and their concentration was measured colorimetrically at 550 nm. The measured absorbance is proportional to the number of living cells, expressed as a percentage of the control.

Cell proliferation (CyQuant) assay

A proliferative cell (reflecting the proportion of viable cells) was monitored using the fluorescent CyQuant detection assay. After removing the supernatant, the plate was placed at -80 °C. After thawing, the cells were incubated with a working reagent prepared according to the manufacturer's protocol, and the fluorescence intensity was detected at 480 nm excitation and 520 nm emission wavelengths using a FlexStation II 384 spectrofluorometer.

Examination of apoptosis

The MitoProbe DilC1 (5) Assay Kit was used to determine the apoptotic process. Decreased mitochondrial membrane potential is considered an early sign of apoptosis on which the procedure is based. On the day of measurement, cells were incubated for 30

minutes at 37 °C with MitoProbeTM DilC1(5) working reagent. After excitation at 630 nm fluorescence was detected at 670 nm using a FlexStation II 384.

Investigation of necrotic processes

SYTOX™ Green Nucleic Acid Labeling Stain was used to detect necrosis-induced cell death. The essence of the procedure, that due to the size of the dye, it is only able to penetrate the cell through the fragmented plasma membrane and bind to nuclear DNA, resulting in a significant increase in fluorescence intensity. After removing the supernatant and washing the cells, the cells were incubated with 1 μM SYTOX™ Green reagent for 30 minutes at 37 °C. Fluorescence was determined at an excitation wavelength of 545 nm and an emission wavelength of 590 nm using a FlexStation II 384 instrument.

TRPV3 RNA interference

The cells were transfected with small interfering RNA specific for TRPV3 and using Lipofectamine 2000 Transfection Reagent at 70% confluence. As a control, cells were transfected with RNAi Negative Control double-stranded siRNA (scrambled siRNA). The decrease in RNAi-induced protein expression was monitored daily by Western blot for four days. The optical densities of the immunoreactive bands were normalized to the β-actin signal and normalized to scrambled RNAi-treated cells.

Statistical analysis

Our measurement results are expressed as mean ± SE and as a percentage of the control group. The standard error of the mean (SEM), also expressed as a percentage of control, is shown in the error bar. The difference between the means was examined by

paired t-test for related samples and by one-way analysis of variance (ANOVA) for more than two groups, using the Bonferroni post hoc test for multiple comparisons and the Dunett post hoc test when comparing the distribution to the control group. SPSS 20.0 statistical software was used for the analyzes. Differences below the 0.05 significance level were considered significant ($p < 0.05$).

RESULTS

TRPV3 is expressed in human skin and in primary epidermal keratinocyte culture

In our experiments, we first determined TRPV3 expression in human skin using full-thickness normal punch biopsies from healthy patients. We showed by immunohistochemistry that TRPV3 is present primarily in the outermost epidermal layer of the skin, within which TRPV3 shows homogeneous staining in the stratum basale and stratum corneum layers. Consistent with these data, strong TRPV3 immunoreactivity is observed in normal human epidermal keratinocyte (NHEK) cells by immunocytochemistry and by Western blot in both preconfluent (80%) and postconfluent (PC2) cells. In addition, we have successfully demonstrated TRPV3 mRNA expression in healthy human skin and in isolated and cultured pre- and post-confluent primary epidermal keratinocytes using the qPCR technique.

TRPV3 acts as a Ca^{2+} -permeable ion channel on normal human epidermal keratinocytes

Subsequently, the functionality of the TRPV3 channel on epidermal keratinocytes was examined. The whole-cell configuration of the patch-clamp technique was used to examine the channel function, and a ramp protocol was used to examine the membrane currents. In the control case, an outward rectifying current was measured with an average reverse potential of -46.1 ± 1 mV (mean ± SEM, n = 6). Current measurements were then performed at four points in the ramp protocol (-90, -40, +40, and +90 mV), and the data were normalized to the current cell membrane capacity in each case. Interestingly,

treatment of the TRPV3 agonist carvacrol at a concentration of 100 μM significantly ($P < 0.05$) increased both inward and outward currents, an effect that was reversible.

To further demonstrate the functional activity of TRPV3, we started from the well-known property that TRPV3 (like most TRP channels) is a mostly (but not exclusively) Ca-permeable channel. Using a fluorescence-based Ca^{2+} -imaging technique, we found that TRPV3 agonists significantly increased the $[Ca^{2+}]_{IC}$ of cells in a dose-dependent manner. Significantly, the reduction in $[Ca^{2+}]_{EC}$ (from 1.8 mM to 0.02 mM) almost completely abolished the $[Ca^{2+}]_{IC}$-increasing effect of TRPV3 agonists. These findings were also supported by siRNA silencing of the *TRPV3* gene significantly reduced carvacrol-induced Ca signaling.

Our results from these measurements support the fact that the TRPV3 ion channel is indeed expressed in a functionally active form on primary epidermal keratinocytes and acts primarily as a non-selective cation channel permeable to Ca^{2+} in the plasma membrane of cells, similarly to previously described human outer root sheath keratinocytes and mouse epidermal keratinocyte populations.

TRPV3 activation inhibits proliferation and induces cell death on normal human epidermal keratinocytes

Subsequently, the cellular effects of TRPV3 activation on growth and survival in human epidermal keratinocyte cultures were investigated. Cells were treated with plant-derived carvacrol and synthetic 2-APB TRPV3 agonists, both of which were found to reduce the viability and proliferation of NHEK cells in a dose-dependent manner. The TRPV3-specific effects of carvacrol and 2-APB were monitored and evaluated using an

RNA interference technique, which showed that successful gene silencing of TRPV3 was able to mostly counteract the aforementioned adverse effects. Based on our results, we can conclude that these agonists indeed modify cell viability and growth through a TRPV3-mediated pathway.

Combined $DilC_1(5)$ - SYTOX Green staining was used to examine the exact form of cell death. We found that both carvacrol (300 μM) and 2-APB (100 μM) at high concentrations significantly reduced mitochondrial membrane potential (an early sign of apoptosis) but did not cause SYTOX Green accumulation. Based on these, it can be stated that activation of the TRPV3 ion channel leads to apoptotic cell death. The use of calcium-binding EDTA did not affect the observed effects, suggesting that this effect of carvacrol and 2-APB is independent of Ca^{2+} mobilization from the extracellular space.

TRPV3 activation elicits a strong proinflammatory response

Because 24-hour treatments reduce cell viability and proliferation, the next step was to investigate the cellular effects of TRPV3 activation in short-term experiments. Changes in the expression of key proinflammatory cytokines (IL-1α, IL-6, IL-8 and tumor necrosis factor-α (TNFα)) were monitored. In our experiments, we found that TRPV3 agonists significantly increased the expression of proinflammatory cytokines and induced a remarkable release of IL-6 and IL-8 on epidermal keratinocytes. These effects were almost completely prevented by selective silencing of TRPV3, indicating that the proinflammatory effects of carvacrol and 2-APB are indeed mediated through TRPV3 activation.

TRPV3-induced proinflammatory effects affect the NF-κB signaling pathway

Next, we aimed to identify the intracellular signaling pathway through which the proinflammatory effects of TRPV3 activation may occur. Activation of the NF-κB pathway is known to play an important role in the regulation of inflammatory processes, so we investigated this mechanism. Treatment with carvacrol for 30 minutes induced (hence inactivated) phosphorylation of the inhibitor IκBα and induced (and thereby activated) phosphorylation of the p65 NF-κB isoform. Both of these effects could be prevented by concomitant use of EDTA.

One feature of the activation of the NF-κB pathway is the translocation of p65 into the nucleus, which was also examined by immunocytofluorescence on primary epidermal keratinocyte cells. Compared with the untreated control, 300 μM carvacrol treatment significantly enhanced nuclear staining, similar to the TLR-3 agonist poly I:C, which is used as a positive control in NF-κB translocation inducer in keratinocytes.

Taken together, our results to date suggest that the NF-κB signaling pathway plays a crucial role in TRPV3-activated inflammatory processes in human epidermal keratinocytes.

Because the NF-κB pathway is known to promote cell survival in keratinocytes, and our results demonstrated that TRPV3 activation reduces cell proliferation and viability, we wanted to investigate the role of the NF-κB pathway in these processes. To answer our question, we examined whether the BAY11-7085 NF-κB inhibitor affects our previous results. The use of the inhibitor in combination with the TRPV3 activator carvacrol further reduced cell viability. This suggests that activation of TRPV3 activates

more than one signaling pathway and that activation of NF-κB partially offsets the negative effect of carvacrol on viability.

TRPV3 expression was significantly elevated in tissue samples from AD patients

In the next phase of our experiments, we wanted to investigate the role of TRPV3 in one of the most common inflammatory skin diseases, atopic dermatitis (AD). In our studies, we placed special emphasis not only on the examination of lesional areas, but also on the non-lesional areas of patients.

We were the first to perform TRPV3 immunostaining on lesional (AD-L) and non-lesional (AD-NL) skin areas in AD patients and on punch biopsy samples from healthy volunteers. As in previous studies, we found that TRPV3 protein expression was significantly higher in lesional areas compared to healthy controls, and also that it was higher in non-lesional areas. In addition, although not significantly, an upward trend in TRPV3 expression was observed in the non-lesional area compared to healthy control samples.

TRPV3 is overexpressed in "epidermal tissue" of AD patients

In our work, we were able to obtain samples from 3 AD patients under local anesthesia by removing the epidermal layer with shave biopsy, from both the lesional and unaffected skin areas, as well as from 3 healthy donors. These samples were divided into 3 parts: two parts were processed immediately and quantitative protein and mRNA expression was determined from “epidermal tissue” by Western blot and qPCR, while *in vitro* epidermal keratinocyte cultures were prepared from the third part. Subsequently,

additional molecular (Western blot and qPCR) and functional (patch-clamp) assays were performed on the cultured cells to more accurately map TRPV3 expression.

qPCR analysis revealed that TRPV3 mRNA expression was significantly higher in lesional tissue in all three donors compared to both healthy and non-lesional epidermal tissue. It was significantly higher in non-lesional tissue in 2 donors, while in 3 cases there was an increasing trend in TRPV3 mRNA expression compared to healthy controls. Interestingly, by Western blotting, TRPV3 protein expression was below the detection level in healthy donors. In contrast, we were able to detect a strong signal from tissue samples from all three patients with AD who showed significantly higher TRPV3 protein expression in the non-lesional regions compared to the affected regions. To our knowledge, this is the first experimental result to show that TRPV3 is overexpressed not only in the lesional but also in the non-lesional epidermis.

TRPV3 also shows elevated expression at the cellular level in keratinocytes from AD patients

Our new results were confirmed by *in vitro* experiments, in which we worked with pre- and post-confluent cultures, similar to our previous experiments. Interestingly, in preconfluent (proliferating) cultures, TRPV3 mRNA expression was significantly higher in 1 donor compared to the healthy control, but not in AD-HEK cells, and in the other 2 donors both AD-NHEK and AD-HEK cells had significantly lower mRNA expression compared to healthy keratinocyte cultures. In contrast, there was significant contrast in post-confluent, differentiated cultures (which model *in vivo*-like conditions in the epidermis much more than in preconfluent cultures) that TRPV3 mRNA expression was

significantly higher in AD-NHEK cells in all three donors and AD -HEK in donors 2/3 as in healthy keratinocytes. Furthermore, TRPV3 expression was significantly higher in lesional keratinocytes than in non-lesional cells in 2 donors, while it was significantly lower in non-lesional and healthy keratinocytes in donor 3.Interestingly, in contrast to Western blot results obtained from tissue samples, healthy epidermal keratinocyte cultures express significant amounts of detectable protein-level TRPV3, but more importantly, this expression level did not show a statistically significant difference between lesional and non-lesional samples in AD patients. regardless of their proliferative (preconfluent) or differentiated (post-confluent) status.

TRPV3 also shows functionally significant hyperfunction in patients with AD

To demonstrate that TRPV3 is also functionally overexpressed in AD skin, we sought to perform patch-clamp experiments on healthy, lesional, non-lesional epidermal keratinocytes. Using the whole-cell configuration of the patch-clamp technique, as well as the results obtained on the primary epidermal keratinocytes described previously, we confirmed and extended these to AD cultures in our present studies. Unfortunately, we were unable to generate a stable current on AD-HEK cells (despite trying more than 150 cells from 3 different donors), presumably due to the increased fragility of these keratinocyte cells. However, on non-lesional keratinocytes, similar to healthy keratinocytes, we found that both inward and outward currents were significantly increased ($p<0.05$) with the TRPV3 agonist 100 μM carvacrol, an effect that was reversible after thorough washout. Currents were measured at 4 different membrane potentials (-90, -40, +40, and +90 mV) and normalized to the current cell membrane

capacity for both NHEK and AD-NHEK. Statistical analysis of currents showed a significant current in both cell types at +40 and +90 mV, whereas this change was significant at -40 and -90 mV only on NHEK cells. Most importantly, carvacrol-induced normalized currents were significantly higher (-90 and +90 mV) in AD-NHEK cells than in NHEK cells, suggesting that TRPV3 is functionally overexpressed in non-lesional cells, compared to healthy keratinocytes.

DISCUSSION

Transient receptor potential vanilloid-3 (TRPV3) is a non-selective, mostly calcium-permeable “thermo” TRP channel with a natural activator in the physiological heat range (33-37 °C). It functions similarly to other TRPV channels, i.e., a complex cellular receptor that is activated by a number of chemical and naturally occurring agents. Recent research has shown that the phenotype of DS-Nh mice and patients with Olmsted's syndrome is due to mutations in the *TRPV3* gene. The latter is characterized by severe skin disorders such as palmoplantar keratoderma, perioroficial hyperkeratosis, diffuse hair loss, baldness, and pruritus. Previous studies on TRPV3 have focused primarily on the observation of mice, in which it has been described that (i) the “gain-of-function” mutation in TRPV3 shows higher Ca^{2+} incorporation and neural growth factor production compared to DS mice without the mutation; (ii) 2-APB activates keratinocytes from DS-Nh mice even at lower concentrations than control keratinocytes; and (iii) TRPV3 activation leads to TSLP release in DS-Nh mice compared to control TRPV3 knockout mice. In humans, TRPV3 expression has been shown to be increased in inflamed (lesional) keratinocytes in AD patients. Although clinical symptoms and the DS-Nh mouse phenotype may be associated with a gain-of-function mutation in TRPV3, which clearly suggests that the channel plays a central role in skin homeostasis, to the best of our knowledge, there is currently no study that investigates the human TRPV3 channel at the molecular level. Our results try to fill this particular gap.

In our experiments, we demonstrated that TRPV3 is expressed at both protein and mRNA levels in human epidermis and on primary human epidermal keratinocytes, and

that the channel is functionally present on keratinocytes and acts as a Ca^{2+} -permeable channel. Since calcium growth has ceased in the absence of $Ca^{2+}_{[EC]}$, it is likely that TRPV3 acts as a membrane channel. To the best of our knowledge, previous studies have not yet reported that TRPV3 is expressed and present as a functionally active membrane channel on primary human epidermal keratinocytes. Activation of the channel with carvacrol or 2-APB not only induced an increase in $Ca^{2+}_{[IC]}$ but also reduced cell viability and proliferation through induction of apoptosis. These effects were significantly preventable by gene silencing of TRPV3 by RNA interference, suggesting that the processes are predominantly TRPV3 dependent. Taken together, these results are consistent with previous results from our group on hair follicles in which TRPV3 activation induced apoptosis and catagen regression, and similar effects were observed on outer root sheath keratinocytes.

A well-documented process in the literature is the production of inflammatory mediators by keratinocytes that connect the epidermis to the more immunologically active dermal parts in general. In our work, we successfully demonstrated that short-term treatment of epidermal keratinocytes with a TRPV3 agonist induced the production and release of proinflammatory cytokines. In addition, our experiments range from epidermal TRPV3 to immune cell activation processes, by showing that the induction of proinflammatory mediator production in keratinocytes depends on NF-κB activation and translocation during several inflammatory processes. In our experiments, we also successfully demonstrated that inflammatory mediator production is mediated through activation of the NF-κB signaling pathway.

Overall, our results support the presence of the TRPV3 ion channel in a functionally active form on human epidermal keratinocytes, where their activation reduces cell viability and induces proinflammatory mediator production via the NF-κB signaling pathway. In addition, our results may trigger further (preclinical and clinical) studies that reveal agents with pharmacological therapeutic potential for TRPV3 (most notably those that induce ion channel inhibition and / or down-regulation) in the treatment of inflammatory skin diseases such as AD- or in dermatitis caused by allergens, irritants or other stimuli.

After our results highlighted that the activation of the TRPV3 channel by carvacrol plays a role in the regulation of the production of inflammatory mediators on human epidermal keratinocytes, we extended our experiments to not only healthy but also to atopic dermatitis, one of the most common inflammatory skin diseases. In our experiments, we also had the opportunity to examine full-thickness skin, epidermal tissue samples, and primary epidermal keratinocyte cultures.

A recent study examined the role of TRPV3 in healthy, non-lesional and lesion-specific AD and psoriatic skin. Using RNA sequencing, it was found that TRPV3 transcripts were increased in the affected skin area of both skin diseases, whereas statistically insignificant changes were observed in the unaffected skin areas in these patients. In the same manuscript, the authors found higher TRPV3 expression in lesional AD skin, supporting previous studies by others.

Several conclusions can be drawn from the novel findings of our present results. In fact, our group provides the first evidence that TRPV3 shows increased expression not

only in the lesional epidermis of AD patients but also in the seemingly healthy non-lesional epidermis (compared to healthy skin), which differs significantly in terms of terminal differentiation and certain immune dysfunctions from normal skin. Elevated TRPV3 levels were also detectable at the protein and mRNA levels in non-lesional and lesional AD skin.

As with virtually all human samples, we found significant variability between donors in different tissue samples (full-thickness skin, epidermal tissue sample, and primary keratinocyte cultures) and in the experimental methods used (immunohistochemistry, Western blot, qPCR). Therefore, we have supplemented the expression analyzes with functional methods (patch-clamp technique), which is one of the most excellent instrumental technologies that can provide real evidence for mapping the more accurate function of an ion channel. Perhaps our most important finding was that agonist-activated TRPV3-channel-mediated ion currents were significantly increased in cultured non-lesional AD keratinocytes compared to currents measured in healthy keratinocytes, which data strongly support the key message of our study.

Summarizing our results, TRPV3 expression is increased not only in the affected but also in the unaffected AD skin areas, thus providing valuable preclinical data for translational and clinical studies aimed at channel modification (most likely inhibition) of atopic dermatitis and possibly other in the treatment of skin diseases.

SUMMARY

In the first part of our experiments, we investigated the expression of transient receptor potential vanilloid-3 (TRPV3) ion channel and its role in inflammation on human epidermal keratinocytes. We identified TRPV3 expression both on human skin and cultured epidermal keratinocytes. We have succesfully demonstrated, that the channel functions as a Ca^{2+}-permeable ion channel; activation with carvacrol and 2-APB, respectively, in dose-dependent manner suppresses proliferation of epidermal keratinocytes and induces cell death through apoptosis. All these effects were shown to be TRPV3-specific, since TRPV3-specific gene silencing antagonized these actions. Short-term activation of the channel triggers a strong proinflammatory response, presumably dependent on the NF-κB signaling pathway.

Subsequently, we aimed to determine the molecular and functional expression of TRPV3 in non-lesional skin of AD patients, which markedly distinct from normal skin with respect to terminal differentiation and certain immune function abnormalities. In our studies, we provide the first evidence that the expression of TRPV3 in full-thickness skin and epidermal shave biopsy samples is significantly increased in non-lesional human AD epidermis, similar to lesional AD samples. Of further importance, we also demonstrated by patch-clamp that TRPV3 is expressed in a more functionally active form on non-lesional AD keratinocytes compared to healthy keratinocytes, as evidenced by the significantly higher (induced by agonists) TRPV3-specific ion current.

Our results highlight that TRPV3 plays an important role in the regulation of inflammatory processes of the skin, thereby it could be a potential therapeutic target in inflammatory skin diseases.

APPENDIX

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Registry number: DEENK/57/2022.PL
Subject: PhD Publication List

Candidate: Nikolett Molnárné Vasas
Doctoral School: Doctoral School of Molecular Medicine

List of publications related to the dissertation

1. **Molnárné Vasas, N.**, Pénzes, Z., Kistamás, K., Nánási, P. P., Molnár, S., Szegedi, A., Szöllősi, A. G., Bíró, T.: Transient Receptor Potential Vanilloid 3 Expression Is Increased In Non-Lesional Skin Of Atopic Dermatitis Patients.
   Exp. Dermatol. [Epub ahead of print], 2022.
   DOI: http://dx.doi.org/10.1111/exd.14530
   IF: 3.96 (2020)

2. Szöllősi, A. G., **Molnárné Vasas, N.**, Angyal, Á., Kistamás, K., Nánási, P. P., Mihály, J., Béke, G., Lisztes, E., Szegedi, A., Kawada, N., Yanagida, T., Mori, T., Kemény, L., Bíró, T.: Activation of Transient Receptor Potential Vanilloid 3 Regulates Inflammatory Actions of Human Epidermal Keratinocytes.
   J. Invest. Dermatol. 138 (2), 365-374, 2018.
   DOI: http://dx.doi.org/10.1016/j.jid.2017.07.852.
   IF: 6.29

List of other publications

3. Orbán-Kálmándi, R. A., Szegedi, I., Sarkady, F., Fekete, I., Fekete, K., **Molnárné Vasas, N.**, Berényi, E., Csiba, L., Bagoly, Z.: A modified in vitro clot lysis assay predicts outcomes and safety in acute ischemic stroke patients undergoing intravenous thrombolysis.
   Sci. Rep. 11 (1), 1-14, 2021.
   DOI: http://dx.doi.org/10.1038/s41598-021-92041-1
   IF: 4.379 (2020)

4. Szegedi, I., Orbán-Kálmándi, R. A., Nagy, A. C., Sarkady, F., **Molnárné Vasas, N.**, Sik, M., Lánczi, L., Berényi, E., Oláh, L., Crișan, A., Csiba, L., Bagoly, Z.: Decreased clot burden is associated with factor XIII Val34Leu polymorphism and better functional outcomes in acute ischemic stroke patients treated with intravenous thrombolysis.
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5. Szöllősi, A. G., Gueniche, A., Jammayrac, O., Papp, J., Blanchard, C., **Molnárné Vasas, N.**, Andrási, M., Juhász, I., Breton, L., Bíró, T.: Bifidobacterium longum extract exerts pro-differentiating effects on human epidermal keratinocytes, in vitro.
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Eur. J. Pharm. Sci. 69, 61-71, 2015.
DOI: http://dx.doi.org/10.1016/j.ejps.2015.01.002
IF: 3.773

Total IF of journals (all publications): 24,25
Total IF of journals (publications related to the dissertation): 10,25

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on the basis of the Journal Citation Report (Impact Factor) database.

24 January, 2022

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