Ph.D. THESIS

ROLE OF THE CAPSAICIN-SENSITIVE NERVE ENDINGS AND TRANSIENT RECEPTOR POTENTIAL VANILLOID 1 (TRPV1) RECEPTOR IN THE PATHOMECHANISM OF ORAL LICHEN PLANUS

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INTRODUCTION

Lichen and lichenoid reaction

Oral lichen planus (OLP) and lichenoid reactions are chronic inflammatory lesions of the oral mucosa. Lichen oris is a form of lichen ruber planus. Lichen ruber planus may be presented as oral or genital involvement. Oral involvement occurs in 50% of lichen ruber planus patients. In 25% of all cases oral mucous membrane alterations are the only symptoms. Oral symptoms may develop prior to dermatological ones [51]. Both are more common in women. Most of the patients are between 30-60 years of age. Pathomechanism of the disease is not clear, autoimmune mechanisms have been favoured recently. T-cell mediated processes may be important with increased production of $T_{helper}1$ cytokines (interferon gamma: IFNγ and tumor necrosis factor alpha: TNF-α) thus leading to apoptosis of basal epithelial cells [48]. Presence of anti-keratinocyte autotoxic T-cells was described in tissue samples by Sugerman et co-workers. The antigen is possibly the heat-shock protein [55]. Development of OLP is preceded by the appearance of certain antigens shown by the decreased expression of the T limphocyte oligo- or monoclonal activation marker vβ [76]. These mechanisms lead to clinical occurrence of the disease with various grades of cell proliferation and apoptosis. The increased apoptosis of basal epithelial cells may be caused by activated CD8+ cells. Supposed mechanisms initiating keratinocyte apoptosis are the following:

1. TNF-α from macrophages acting on TNF-αR1 receptors on epithelial cells
2. CD95L expressed on T-cells binding to CD95 on epithelial cells
3. Granzime B of T-cells induces apoptosis [54].

However, other factors cause cell proliferation. CD8 positive cells also produce macrophage-migration inhibition factor and RANTES factor [75]. RANTES factor induces increased cell proliferation. If the rate of apoptosis exceeds the rate of proliferation, the clinical presentation tends to be atrophic, later on erosive. If the rate of proliferation rate exceeds that of apoptosis, reticular, papulosus, plaque types develop [10,75].

The connection of diabetes and OLP has been shown by Greenspan and co-workers in 1966 [21] and has been confirmed by other studies [29,43]. Patients suffering from diabetes often develop atrophic and erosive types of OLP [9]. Malfunction of phagocytes, as well as increased production of cytokines (e.g. a TNF-α) have been reported in diabetes. Rise of collagenase activity results in more severe inflammation and decreased regeneration [67].

[Ide írhatja a szöveget]

Psychiatric factors (neurosis) and hypertonia may also play a role in OLP. Recently bacterial and viral factors have been suggested, but have not been proved unequivocally. Human papilloma virus (HPV), an epitheliotropic DNA virus, can contaminate squamous epithelial cells of skin and mucous membranes. DNA from HPV can be found in OLP lesion, just like in clinically intact mucous membranes [56]. Hepatitis C virus has several extra-hepatic symptoms including OLP [13]. In some countries (e.g. in Italy) hepatitis C virus associated OLP is associated with HLA-DR6 [12]. HCV positive hepatitis is mainly associated with the red forms of OLP.

Lichen planus is mostly bilateral [41], whilst lichenoid reaction is unilateral. However, unilateral lichen planus and bilateral lichenoid reaction cases are known. Both are atrophic or erosive lesions covered by papule. The two forms can not be distinguished clearly. According to the classification of Lodi and co-workers oral lichenoid reactions are lesions that partially fit the histological criteria of OLP [36]. Oral lichenoid reactions may be precipitated by dental reconstructive materials (amalgam, golden- or silver-palladium-alloy, composites). Contact allergy may underlie this phenomenon. Graft versus host disease following bone marrow transplantation is often accompanied by the symptoms of oral lichenoid reaction [47]. Aromatic compounds (cinnamon, menthol, ammonium chloride) in chewing gums, tooth paste and mouthwash may also evoke oral lichenoid reactions.

Different clinical manifestations of OLP were first identified by Andreasen in 1968 [1]:

- Reticular (lichen oris reticularis)
- Annular (lichen oris annularis) [70].
- Papulosus (lichen oris papulosus)
- Plaque
- Atrophic (lichen planus atrophicus)
- Erosive or ulcerative (lichen planus ulcerosus)
- Bullosus (lichen planus bullosus)
- Pigmentosus (lichen oris pigmentosus)

Clinical appearance of OLP is variable. It is important to clearly distinguish it from similar symptoms caused by other disorders.

World Health Organization (WHO) released histological classification of OLP and lichenoid reactions in 1978 and 1997. Histologically OLP involves subepithelial lymphocyte infiltration, ortho- and parakeratosis, acanthosis of the epithelium. In case of lichenoid

reaction diffuse and deep inflammation is more characteristic. Presence of plasma cells and eosinophils is diagnostic for lichenoid reaction. Both lesions show dissolution of basal epithelial cells and epithelial hyperkeratosis. Inflammatory cells and tissue antigens are involved in both conditions. The role of neurogenic factors is still debated in these disorders.
Chronic inflammation modulates the release of neurotransmitters from peripheral sensory nerve endings which may be related to long term processes (e.g. contraction, destruction, formation and granulation of nerve fibres). Loss of peripheral innervation may inhibit normal growth and regeneration of mucous membrane leading to ulceration. Local inflammatory mediators from inflammatory cells can activate sensory nerves forming a positive feedback loop to sympathetically mediated inflammation and pain.

Mechanism of neurogenic inflammation

The main function of sensory nervous system is sensing stimuli of the inner and outer environment by peripheral nerve endings and forwarding them to the central nervous system by sensory fibres. Stricker discovered in 1876 that antidromic electrical stimulation of dorsal root ganglia leads to vasodilatation in the skin. This phenomenon was called „antidromic vasodilatation" [6]. He suggested that mediators responsible for antidromic vasodilatation may be released from special effector endings of certain primary afferents. It is proven now that some sensory axon terminals have both afferent and efferent functions. These terminals can release neuropeptides stored in vesicles without axonal conduction in response to stimuli [38,64]. There are afferent only and dual function sensory afferents in the spinal cord and selectively efferent sensory neurons are assumed [28]. Calcitonin gene-related peptide (CGRP) released from sensory nerve terminals causes vasodilatation whereas tachykinins (e.g. substance P: SP, neurokinin A: NKA and neurokinin B: NKB) lead to plasma protein extravasation, mast cell activation and leukocyte accumulation at the innervated site. These mechanisms are known as neurogenic inflammation [40,45]. Neurogenic inflammatory components play an important role in the pathomechanism of several diseases (e.g. rheumatoid arthritis, bronchial asthma, psoriasis, eczema, allergic contact dermatitis and inflammatory bowel diseases [37,62]). Present anti-inflammatory drugs are unable to inhibit neurogenic processes of these inflammatory reactions [26].
It was discovered by the research group of the Department of Pharmacology and Pharmacotherapy, University of Pécs that somatostatin is also released from activated sensory nerve terminals Somatostatin reaches the circulation evoking systemic anti-inflammatory, and anti-nociceptive effects. This is the third function of the sensory nerve endings [25,27,60,61].

This systemic efferent action has been denominated as „sensocrine function" by Professor Szolcsányi [59].

Capsaicin

Hot pepper is active in America. It was called chilli by the Aztecs and is still known by this name throughout Latin-America. The scientific name of chilli pepper (*Capsicum annuum*) has been given by de Tournefort, a French botanist.

The pungent compound of hot pepper was first isolated by Thresh in 1846 [69]. He also showed that the chemical structure of capsaicin and vanillin is similar. The chemical structure of capsaicin, 8-methyl-N-vanillyl-6-nonenamide, was identified in 1919, however the synthesis has only succeeded 20 years later.

Capsaicin was widely used for hundreds of years in traditional medicine for the treatment of joint inflammation, as well as for analgesia. Capsaicin containing ointments or bandages are capable of easing spinal pain, depressing rheumatoid joint inflammation and the signs of sensory neuropathy. Native Americans have used the legume of hot pepper for toothache. In 1850 a paper has been published in Dublin Free Press on therapeutic use of capsaicin suggesting alcoholic extracts of hot pepper for toothache [7].

TRPV1 receptor

Existence of a capsaicin receptor was first suggested by János Szolcsányi and Aranka Jancsó-Gábor in 1975. They examined the nociceptive effects of capsaicin and other vanilloid analogues in rats. Based on the tight structure-effect correlations they concluded that capsaicin evokes its effect through receptorial mechanisms. They also suggested a hypothetic structure of the receptor [65,66]. Twenty years later the gene encoding the receptor was identified and structure of the receptor was elucidated. It has been proven with patch clamp technique in 1990 that capsaicin and resiniferatoxin opens the same cation channel on the plasma membrane of sensory neurons [8]. Capsaicin receptor has been cloned in 1997 by Julius and co-workers and denominated as vanilloid receptor 1 (VR1) because it can be activated by vanilloid compounds (e.g. resiniferatoxin) [15]. According to the structure of the receptor this name was later changed as the receptor was classified to transient receptor potential (TRP) superfamily as the first member of vanilloid family: transient receptor potential vanilloid 1, TRPV1 [23]. Interestingly, other members of the TRPV superfamily (TRPV2-6) are not sensitive to vanilloid molecules, they are vanilloid receptor-like structures (VRL) and epithelial Ca^{2+}-channels (ECAC) using their old nomenclature [23].

Human TRPV1 receptor shows 92% sequence homology with the rat TRPV1 [24,39]. The receptor consists of 838 amino acids forming 6 β-layer domains. The channel region is an intracellular, hydrophobic loop between the 5th and 6th subunit. These structures form tetramer-like non-cation selective ion channels in the plasma membrane. On the activation of the receptor intracellular Na^+ and Ca^{2+} ion levels increase, while K^+ level decreases. Na^+ is mainly responsible for generation of action potential discharge leading to nociception and pain sensation. Increase of intracellular Ca^{2+} causes release of sensory neuropeptides from nerve terminals. Due to long term or repeated activation elevated concentration of cations induce swelling of the cytoplasm and mitochondria. As long term consequence energy production of the cell decreases and cell function fails. This process is the molecular background of desensitisation by high doses of capsaicin. Function of the axon terminals remains damaged for months [8,26,63].

TRPV1 is a polymodal sensory ion channel. It can be activated by intra- or extracellular physical or chemical stimuli. TRPV1 can be activated by painful heat over 43ºC [68,72] and proton concentrations under pH 6. There are several vanilloids derived from plants such as resiniferatoxin (RTX) from the Mexican cactus-like plant (*Euphorbia resinifera*), piperin from pepper (*Piper nigrum*), zingeron from ginger (*Zingiber officinale*) that can open the cation channel. Interestingly, capsaicin due to its lipophilicity binds to the intracellular part of the receptor [42]. There are various endogenous ligands of the receptor (e.g. endocannabinoid N-arachidonyl-ethanol-amine (AEA), also known as anandamide [15]; 12-hydroperoxy-eicosatetraenic acid (12-HPETE), or N-arachidonyl-dopamine (NADA). It is also known that bradykinin, which is the strongest pain evoking mediator, acting on B_2 receptors sensitizes TRPV1 via intracellular secondary messenger mechanisms, as well as regulates the synthesis of lipoxigenase products via activation of phospholipase A_2 [44,50]. Action of the channel can be markedly enhanced by prostaglandins, such as prostaglandin E_2, the key molecule in inflammatory processes or prostaglandin I_2 also known as prostacycline [16,57]. The potent endogenous ligand of the receptor is still unknown.

Desensitisation achieved by pre-treatment with repeated high doses of capsaicin or RTX is used to examine the role of capsaicin-sensitive nerve endings in acute inflammatory and pain processes. Using this method a long term loss of function can be reached in the whole terminal [3,4,58].

Besides vascular responses neurogenic inflammation induces other effects, such as positive inotropic and chronotropic effects in the heart, bladder relaxation evoked by CGRP and miosis mediated by tachykinins. SP, NKA released from TRPV1 expressing sensory nerves

cause bronchoconstriction in the guinea pig, whilst it leads to bronchodilatation mediated by nitric oxide (NO)/prostanoids in rats and mice. Similarly to guinea pigs bronchoconstriction occurs in humans [8].

TRPV1 receptors have been described in the peripheral and central nervous systems [14,15]. TRPV1 has been identified in isolated human keratinocytes [30,52], human epidermis [17], mast cells [54], dendritic cells [5], epithelial cells, vascular endothelial cells, glands of the nasal mucosa [49], urothelium [2] and gastric mucosa [20] by immunohistochemistry and PCR.

AIMS OF THE STUDY

I. The aim of our study was to detect TRPV1 receptors by immunohistochemistry and changes of its expression by molecular biological methods in oral mucosa samples of healthy volunteers and oral lichen planus patients.

II. It is known in the literature that amalgam dental fillings may induce lichenoid reaction in the oral mucosa. We decided to examine in a rat model whether mercury chloride ($HgCl_2$) smearing of the oral mucosa is able to induce lichenoid reaction, if there is any change in the expression of TRPV1 receptors compared to normal oral mucosa and whether desensitisation of sensory fibres by capsaicin does influence TRPV1 expression or $HgCl_2$-induced inflammation.

CHAPTER I

INVESTIGATION OF THE EXPRESSION OF TRANSIENT RECEPTOR POTENTIAL VANILLOID 1 (TRPV1) RECEPTOR IN ORAL LICHEN PLANUS

INTRODUCTION

Neuropeptides released from capsaicin-sensitive sensory endings by the activation of TRPV1 receptors exert neurogenic inflammation. TRPV1 receptors were identified on non-neural cells for instance on keratinocytes, inflammatory and immune cells as well as on vascular endothelial cells by immunohistochemistry and PCR, but their functional importance and physiological role have not been elucidated properly. The aim of the present study was to detect the presence of TRPV1 receptors and peripheral expression of receptor mRNA in normal oral mucosa and mucous membrane samples from OLP patients.

[Ide írhatja a szöveget]

MATERIALS AND METHODS

Subjects

Biopsies (0.5 x 0.5 cm) of the oral mucosa have been obtained from 18 patients between 40-60 years of age (5 males and 13 females). The patients gave written informed consent prior to the participation. OLP had been diagnosed on the basis of clinical signs supported by routine histology using haematoxylin-eosin staining. Biopsies were also performed on 5 healthy volunteers between 30-40 years of age (3 males and 2 females). Before excision the oral mucosa was infiltrated with 1 ml lidocaine-adrenaline (2%-0.001%).

Patients involved in the study did not receive local or systemic steroid therapy. The study was approved by the Local Ethics Committee of University of Pécs.

Tissue samples

The excised tissue samples were cut into two. One part was put into RNA-later and stored at -80^{o}C. The other part was fixed in 4% paraformaldehyde and embedded in paraffin for immunohistochemical examination.

Immunohistochemistry

The slides were incubated with rabbit polyclonal anti-TRPV1 antibody followed by horseradish peroxidase (HRP)-conjugated En Vision system anti-rabbit secondary antibody. Immunolocalization of the TRPV1 receptor was visualized by diaminobenzidine (DAB). Nuclear staining was performed with hematoxylin. Semiquantitative scoring of the TRPV1 immunopositivity was performed blind by an expert pathologist. Image analysis was carried out with Image Pro Plus 4.5 software. Immunopositive pixels were counted in the epithelium and connective tissue.

Quantitative Real-Time PCR (qRT-PCR)

QRT-PCR was performed on an ABI Prism 7000 sequence detection system with 5' nuclease assay. Three μg of total RNA were reverse-transcribed into cDNA by 15 U of AMV reverse transcriptase (RT) and 0.025 μg/μl random primers (Promega). PCR amplification was performed with TaqMan primers and probes following the TaqMan universal PCR master mix protocol. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β-actin were used as controls.

Statistical methods

Data are expressed as mean±SEM. One-way ANOVA followed by Bonferroni's post test was used for the calculation of significant differences.

RESULTS

Control buccal mucous membrane showed moderate TRPV1 receptor positivity intracellularly in epithelial cells, compared to the negative control. Epithelial cells showed intracytoplasmic but not nuclear labelling. Immunostaining was observed in all epithelial cell layers from basal to superficial. No reaction was observed in subepithelial tissues. Lymphocytes, fibroblasts and endothelial cells were negative as well.

In lichen planus, intensive immunopositivity was demonstrated in the total thickness of epithelium and submucosal tissues. Pronounced immunostaining was localized in the mucous membrane. Immunostaining of epithelial cells was observed in the basal, parabasal, intermediate and superficial cell layers of the mucous membrane. Immunoreaction was detected in the cytoplasm and some of the cells showed intranuclear positivity, too. Large number of immigrant lymphocytes and fibroblasts were immunopositive in the submucosal region. Intranuclear and intracytoplasmic immunostaining was detected in lymphocytes and fibroblasts. Much more intensive labelling was observed in the capillary endothelial cells of the submucosa.

According to quantitative image analysis percentage of immunopositive pixels was 21.2±8.2% in the selected regions of the control epithelium. It increased to 61.4±4.7% in lichen samples. In spite of numerous immunopositive cells (lymphocytes, fibroblasts and vascular endothelial cells) in subepithelial connective tissue of the lichen samples, quantitative differences of immunostaining can not be detected. The percentage of immunopositive pixels was 14.8±4.9% in the control and 13.9±3.8% in the lichen samples. Quantity of TRPV1 receptor mRNA in samples of lichen oris patients was significantly elevated compared healthy volunteers.

DISCUSSION

In this study we have presented immunohistochemical evidence that expression of TRPV1 receptors is increased in oral lichen planus compared to normal mucosa. On the basis of semiquantitative histological analysis increased specific immunopositivity was observed in the total thickness of epithelium, in vascular endothelial cells, lymphocytes and fibroblasts in

the subepithelial tissue. According to the quantitative image analysis highly increased number of immunopositive pixels was detected in the epithelium dissected from OLP patients compared to normal controls suggesting overexpression of TRPV1 receptor protein in this inflammatory disease. Lymphocytes, fibroblasts and vascular endothelial cells were stained also in the connective tissue but significant differences in the number of immunopositive pixels were not established in lichen and control samples. The virtual inconsistency between the results of semiquantitative scoring and quantititative image analysis can be explained with the fact that the size of the inflamed area was small compared to total extent of connective tissue. Since local expression of these receptors was also proven on mRNA level with quantitative real-time PCR extra-neuronal synthesis of TRPV1 structures is supposed.

Although numerous papers provide evidence that neurogenic inflammation contributes to the pathogenesis of inflammatory diseases (e.g. Sjögren's syndrome, psoriasis, dermatitis, arthritis and asthma), only few reports mention possible neurogenic factors associated with OLP [41]. Proinflammatory neuropeptides, mainly substance P and calcitonin gene-related peptide (CGRP) are thought to be released from the sensory nerve endings via activation of neuronal TRPV1 receptors. These mediators induce neurogenic inflammatory changes, such as local vasodilatation, plasma protein extravasation and accumulation of inflammatory and immune cells [37]. Expression of TRPV1 receptors has also been shown in a wide range of non-neuronal cells such as epithelial cells (keratinocytes [17,30], gastric epithelial cells [20], enterocytes and pneumocytes [22]. Vascular endothelium, immune cells [46], smooth muscle cells [22] and fibroblasts [32] also show TRPPV1 immunopositivity. In spite of the extensive research and numerous immunohistochemical evidence, physiological role of TRPV1 receptors in the non-neuronal tissues still remains elusive [22].

TRPV1 immunoreactivity was shown in human skin samples including the epidermis, hair follicles, skin appendages, dermal blood vessels, Meissner corpuscles, fibroblasts and mast cells [30,32]. Isolated human keratinocytes also express TRPV1 receptors and capsaicin was shown to evoke Ca^{2+} uptake in cultured keratinocytes [30,53]. Furthermore, capsaicin-evoked intracellular Ca^{2+} increase was blocked by TRPV1 antagonist capsazepine. There is some evidence supporting the functional role of TRPV1 receptors. Southall and co-workers suggest that TRPV1 receptors may be sensors for noxious stimuli in keratinocytes and they are overexpressed in inflammation that occurs secondary to epidermal damage [53]. Activation of TRPV1 receptors in keratinocytes was also reported to induce prostaglandin E_2 and

interleukin-8 release as well as increased cyclooxygenase (COX) and matrix metalloproteinase-1 expression [22,35,53].

In our study we provide the first immunohistochemical evidence that TRPV1 receptors are present in human oral mucosa and they are over-expressed in human OLP. Increased number of immunopositive non-neural cells was demonstrated in the total thickness of epithelium and submucosal tissues in oral lichen planus compared to normal mucosa. TRPV1 receptor mRNA level of lichen oris samples was significantly elevated compared to healthy volunteers. Therefore it is supposed that non-neural TRPV1 receptors in oral mucosa may regulate inflammatory changes during development of OLP. Interactions between neural and non-neural TRPV1 receptors via inflammatory mediators (e.g. cytokines, prostaglandins, growth factors and neuropeptides) may be suggested.

Even if the precise pathogenesis of OLP remains still unclear, it is concluded that TRPV1 receptor-dependent release of inflammatory mediators from epithelial, vascular endothelial cells, lymphocytes or fibroblasts may influence the progression of the disease.

CHAPTER II

EFFECT Of NEONATAL CAPSAICIN-DESENSITIZATION ON MERCURY CHLORIDE ($HgCl_2$)-INDUCED LICHENOID REACTION IN THE RAT ORAL MUCOSA

INTRODUCTION

It has been described in the literature that lichenoid reactions (OLR) in the oral cavity are frequently associated with mercury-containing dental amalgam fillings [18]. The mercury component of amalgam has been proposed to be responsible for the inflammatory reactions. Therefore, in order to precisely investigate this pathophysiological mechanism, we have established an animal model. Although the symptoms of lichen planus cannot be reproduced in animals, previous papers reported on the development of lichenoid reaction in response to topical application of mercury chloride [33,34]. It is supposed that pro-inflammatory and anti-inflammatory neuropeptides released by the activation of TRPV1 capsaicin receptors from peptidergic sensory fibres of the oral mucosa modulate the amalgam-induced inflammatory reactions. Recently, non-neuronal TRPV1 receptors have also been shown on keratinocytes but their functional role has not been elucidated yet. The aims of our rat experiments were the following:

[Ide írhatja a szöveget]

1. To investigate the effect of chronic $HgCl_2$ smearing on the oral mucosa.
2. To examine the expression and alterations of TRPV1 receptor both at the mRNA and protein levels.
3. To evaluate the role of capsaicin-sensitive sensory fibres and neurogenic inflammatory components with neonatal capsaicin pre-treatment.

MATERIALS AND METHODS

Animals

Data in literature suggested that the Brown Norway rat strain is the most sensitive to $HgCl_2$ [18]. Therefore these animals were bred and kept in the Animal House of the Department of Pharmacology and Pharmacotherapy, University of Pecs, under climatized conditions and provided with standard rat chew and water *ad libitum*, they were used in our experiments.

Systemic neonatal capsaicin pretreatment

Capsaicin-sensitive sensory fibres were destroyed (desensitized) in newborn Brown Norway rats (2-3 days old, 10-15 g) with 50 mg/kg s.c. capsaicin to exclude neurogenic interactions. Seven weeks old pre-treated and non-desensitized control animals were used in the experiments.

Megjegyzés [HZS1]: A poszterben 30 mg/kg van. Most melyik a jo?

Topical application of $HgCl_2$

The buccal mucosa was painted with 0.5% $HgCl_2$ dissolved in glycerol every other day throughout an 8-week period. The following four experimental groups were investigated:

1. Capsaicin-desensitized and $HgCl_2$-treated.
2. Not capsaicin-pretreated, smeared with $HgCl_2$.
3. Capsaicin-desensitized, non-$HgCl_2$-treated.
4. Not capsaicin-desensitized and not smeared with $HgCl_2$.

At the end of the treatment animals were sacrificed in deep pentobarbital (Nembutal, 50 mg/kg i.v.) anaesthesia and mucosal samples were dissected for histological, immunohistochemical and molecular biological examinations.

Histopathological examination

Histopathological changes were analyzed on sections stained with hematoxylin and eosin. Blind semiquantitative scoring was performed by an independent pathologist. The composite scores were obtained from 4-8 animals/group based on lichenoid histopathological changes

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(e.g. hyperkeratosis, parakeratosis, acanthosis, capillarization, fibroblast proliferation and fibrosis).

Immunohistochemical examination

Expression and localization of TRPV1 receptors in the mucosal tissue were examined with immunohistochemistry. The slides were first treated with polyclonal anti-TRPV1 primary antibody raised in rabbits, then incubated with horseradish-peroxydase-conjugated EnVision system anti-rabbit secondary antibody. The immunolocalization of TRPV1 receptors was visualized with diamino-benzidine (DAB). Nuclear staining was performed with hematoxylin. Specific immunostaining was examined on keratinocytes, vascular endothelial cells, lymphocytes, and fibroblasts.

Quantitative Real-Time PCR (qRT-PCR)

Expression of TRPV1 receptor mRNA in the mucosal tissue was detected by qRT-PCR with the ABI Prism 7000 sequence-detection system using 5' nuclease assay. PCR amplification was performed with TaqMan primers based on the TaqMan universal PCR mastermix protocol. Ribosomal 18S RNA served as internal control.

Statistical analysis

Results are shown as means±SEM. One-way analysis of variance (ANOVA) followed by Bonferroni's modified t-test was used for statistical evaluation. Since the histopathological score values determined with semiquantitative evaluation are non-parametric variables, they were presented as box plots. In this case statistical analysis was performed with Kruskal-Wallis test followed by Dunn's post test.

RESULTS

Lichenoid reactions of different degrees developed in response to the treatments. The keratotic rat oral mucosa became hyperkeratotic, the stratum corneum was much thicker. Topical application of $HgCl_2$ by itself induced lichenoid hyperkeratosis, parakeratosis, acanthosis, and signs of cellular activation were seen in the stratum basale. Pseudo-papillomatosus epithelial hyperplasia could also be observed in some rats. This $HgCl_2$-induced mucosal lichenoid reaction was more severe in capsaicin-desensitized animals. As a result of contact $HgCl_2$ effect on the epithelium, subepithelial fibroblast activation was only detected in capsaicin-desensitized and $HgCl_2$-treated animals. In this group, subepithelial proliferation of capillaries and mild fibrotic reaction developed in the subepithelial layer.

[Ide írhatja a szöveget]

Neither lymphoid reaction, nor hydropic degeneration was present in the basal layer of the oral mucosa in this rat model.

Besides the qualitative examination, composite semiquantitative histopathologial score based on lichenoid histopathological changes also revealed that $HgCl_2$-treatment induced a lichenoid reaction in the rat oral mucosa. Systemic neonatal capsaicin pre-treatment evoked a minimal inflammatory reaction in adult rats, but significantly increased the $HgCl_2$-induced lichenoid symptoms.

Enhanced immunolocalization of TRPV1 receptor protein was detected in the epithelial layers after neonatal capsaicin-pretreatment. Topical $HgCl_2$ application only induced a minimal increase of the TRPV1 immunostaining. $HgCl_2$ treatment did not further increase the TRPV1 receptor immunopositivity in the keratinocytes compared to the effect of capsaicin-desensitization by itself. Clear TRPV1 immunopositivity was seen in the capillary epithelial cells after $HgCl_2$ application, which was more intensive in the capsaicin-pretreated group. Fibroblasts did not express TRPV1 receptors in the mucosa of intact animals. In contrast, in response to only $HgCl_2$ exposure, these cells showed mild TRPV1 immunostaining which increased following capsaicin-desensitization. Accumulated lymphocytes did not express the TRPV1 receptor in any of the experimental groups. The composite score indicating TRPV1 receptor immunopositivity in epithelial cells, capillary endothelial cells, lymphocytes and fibroblasts revealed that systemic neonatal capsaicin-pretreatment markedly, topical $HgCl_2$ painting only minimally increased the number of the TRPV1-immunopositive cells in the rat oral mucosa. It was most surprising that neonatal capsaicin-desensitization by itself increased epithelial TRPV1 expression. Dual treatment (capsaicin and $HgCl_2$ together) induced similar up-regulation and did not enhance it further.

In accordance with these data, molecular biological results obtained with qRT-PCR measurements provided evidence that relative expression of TRPV1 receptor mRNA increased 5 folds in mucosal samples of capsaicin-pretreated rats compared to intact controls. This was not elevated further after capsaicin plus $HgCl_2$ smearing. $HgCl_2$ by itself did not alter TRPV1 mRNA levels.

CONCLUSIONS

Our results obtained in Brown Norway rats clearly demonstrate in accordance with literature [18] that topical application of $HgCl_2$ on the oral mucosa induces a well-defined and characterized lichenoid reaction. There is hyperkeratosis, parakeratosis, achantosis, and even

pseudopapillomatosus epithelial hyperplasia in some individuals. Capillary proliferation and fibroblast activation were also detected in the subepithelial layer leading to mild fibrosis.

Destruction of the capsaicin-sensitive sensory fibres by neonatal systemic capsaicin pre-treatment increased the severity of $HgCl_2$–induced lichenoid inflammatory signs. Subepithelial fibroblast activation only developed after combined treatment. Therefore, we presume that capsaicin-sensitive sensory neurons exert protective influence on the development of lichenoid reactions.

It is well-described that due to the neurotoxic effect of systemic neonatal capsaicin-pre-treatment function of the capsaicin-sensitive sensory neurones is selectively abolished. It was surprising that neonatal capsaicin-pretreatment markedly increased TRPV1 receptor immunopositivity on keratinocytes, fibroblasts and capillary endothelial cells. Furthermore, quantitative RT-PCR data revealed significantly increased TRPV1 receptor mRNA concentration in these mucosal samples. These results clearly show that neonatal capsaicin-pre-treatment desensitizes neural TRPV1 ion channels, but significantly increases the expression of non-neural receptors. It is possible that this is a consequent adaptation mechanism in response to the chemical denervation. Our observation might be an important milestone in elucidating the physiological function of non-neural TRPV1 receptors. The fact that capsaicin-pretreatment which induced selective desensitization of capsaicin-sensitive nerves increases $HgCl_2$-induced inflammatory changes clearly shows that neuromediators released from these fibres play a protective role in the development of lichenoid reaction. Overexpression of extraneural TRPV1 receptors after capsaicin-pretreatment would contribute to the pathological mechanism of inflammatory processes.

Topical application of $HgCl_2$ by itself only minimally increased TRPV1 immunopositivity and mRNA did not change in the samples.

It can be concluded that in $HgCl_2$-induced lichenoid reaction in the rat oral mucosa, despite its role in human lichen planus, expression of non-neural TRPV1 receptors does not significantly increase.

SUMMARY OF ORIGINAL FINDINGS

1. We provided immunohistochemical evidence in the human oral mucosa that the expression of TRPV1 receptor increases in lichen planus compared to healthy tissues. Enhanced specific immunostaining was observed in the total width of epithelium, in vascular endothelial cells, lymphocytes, fibroblasts and subepithelial connective tissue.

Quantitative picture analysis revealed significantly increased immunopositive pixel ratio in the epithelial samples of patients suffering of OLP compared to nornal, healthy controls suggesing an increased TRPV1 expression during this inflammatory reaction. Lympocytes, fibroblasts and vascular endothelial cells in the connective tissue also showed greater TRPV1 immunopositivity in OLP tissues, but significant difference compared to non-inflamed samples could not be determined with the computerized pixel quantification. This virtual contradiction between the semiquantitative and the quantitative picture analysis might be explained by the fact that the inflamed area compared to the total size of the connective tissue is too small. Since the local expression of the receptors was evidenced with RT-PCR at the mRNA level, it can be supposed that they are synthesized by extraneural structures such as keratinocytes, fibroblasts and lymphocytes.

2. Since desensitization of capsaicin-sensitive sensory fibres by neonatal systemic capsaicin pre-treatment increased inflammatory signs of $HgCl_2$–induced lichenoid reaction in rat oral mucosa, we presume that capsaicin-sensitive sensory neurons exert protective influence on the development of lichenoid reactions. The overexpression of extraneural TRPV1 receptors after capsaicin-pretreatment both at mRNA and protein levels might contribute to the pathological mechanisms of the inflammatory processes.

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PUBLICATIONS RELATED TO THE THESIS

1. **Bán Á.**, Marincsák R., Bíró T., Perkecz A., Gömöri É., Sándor K., Tóth I.B., Bánvölgyi Á., Szolcsányi J., Pintér E: Up-regulation of Transient Receptor Potential Vanilloid Type-1 (TRPV1) receptor expression in oral lichen planus. *Neuroimmunomodulation* 2009 (IF: 1,21)
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