THE ROLE OF CAPSAICIN-SENSITIVE SENSORY NERVE ENDINGS AND THE TRPV1 RECEPTOR IN SCLERODERMA AND CHRONIC ARTHRITIS ANIMAL MODELS

PhD THESIS

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INTRODUCTION

According to the classical theory on neuro-regulation, sensory nerves transmit sensory stimuli and pain from various body parts (joints, skin, internal organs) to the central nervous system. The other group of the peripheral nervous system is responsible for efferent reflexes triggered by incoming stimuli, i.e. motory or vegetative functions.

From neuro-regulation's theoretical point of view **capsaicin-sensitive** sensory nerves represent a special and interesting third group, the peripheral endings of which not only possess 'classical' afferent functions (nociperception), but implement efferent functions as well. Directly, without reflex, certain neuropeptides are released from them, bringing forth forcible vessel dilation, plasma protein emission and inflammatory cell activation on the area of innervation. Epitomised, this phenomenon is called neurogenic inflammation. These inflammatory mediators are calcitonin gene-related peptide (CGRP) which primarily causes vasodilatation, and the tachykinins, e.g.: SP and neurokinin A (NKA), that are responsible for plasma protein extravasation. These processes mediate ***local efferent functions*** of capsaicin-sensitive afferents.

Subsequent studies revealed that from these same activated sensory nerve endings, aside from the inflammatory neuropeptides listed above, somatostatin (SOM) is also released, which has systemic anti-inflammatory and analgetic effects, once getting into the circulation.

This is the third, ***systemic efferent function*** of sensory nerve endings, named *sensocrin effect* by Professor Szolcsányi, following the formula of somatostatin's endocrin and paracrin effect.

Capsaicin (*Capsicum annuum és Capsicum frutescens*) is the prickly substance of paprika, an alkaloid regarding its chemical structure: 8-methyl-N-vanillyl-6-nonenamide. Capsaicin effectuates its impacts via receptorial mechanisms. The capsaicin receptor expressing gene was identified in 1997, and the capsaicin receptor was named Vanilloid 1 receptor (VR1). Later the international nomenclature based on the structure of receptors altered this denomination, the ligand-dependent cation channel was classified into the Transient Receptor Potential (TRP) family, and as number 1 member of the vanilloid family, it was then renamed Transient Receptor Potential Vanilloid 1 (TRPV1).

TRPV1 is a cation channel with polymodal sensor function, which can be activated by several physical or chemical stimuli – e.g.: painful temperature, above 43 ºC and proton concentration below pH 6 – both intra- and extracellularly. When the receptor is activated, Na^+ and Ca^{2+} ions stream in, followed by outflow of K^+ ions from the cell. Inflow of Na^+ ions is mainly

responsible for creating action potential, as a consequence of which nociception, sensation of pain evolves. Influx of Ca^{2+} causes release of neuropeptides from nerve endings.

As a result of enduring or repeated activation the high cation concentration accumulated in the cell causes swelling of cytoplasm and mitochondria, and as a long-term consequence energy circulation of the cells decrease, the nerve ending becomes nonfunctional.

This process gives the molecular basis of desensibilization by pre-treating with high doses of capsaicin. A new perspective came regarding the analysis of receptor TRPV1 when in 2000 two separate research groups generated TRPV1 receptor knockout mice. With the help of these mice, the relevance and functioning of TRPV1 ion channel can be studied selectively in *in vivo* models.

Systemic anti-inflammatory somatostatin released during the TRPV1 receptor activation could be a promising anti-inflammatory and analgetic drug, however the therapeutic use of native somatostatin is inhibited by its wide spectrum and extremely short (less than 3 minutes) plasma elimination half-life. Stabile, selective sst_4/sst_1 agonists can however provide a new therapeutic opportunity in treating inflammation and pain.

The great advantage of these compounds is that they do not possess the endocrine effects mediated by somatostatin's sst_2, sst_3 és sst_5 receptors. The stabile cyclic heptapeptide generated by the MTA Peptidobiochemical Research Group, TT-232 (D-Phe-Cys-Tyr-D-Trp-Lys-Cys-Thr-NH_2), seems a promising drug candidate.

AIMS OF THE STUDY

Skin and joints are especially richly innervated by capsaicin-sensitive sensory nerve fibers, which possess important modulating functions in neurogen processes and processes accompanied by pain. Hence in our experiments we performed the complex study of peptiderg sensory neuron endings, the TRPV ion channels localized on them and the sensory neuropeptides released from them, on scleroderma and arthritis models through biochemical, immunological and morphological methods.

1. In the first section of the study using TRPV1 receptor and CGRP knockout mice, we examined the relevance of TRPV1 receptor and the role of the vasoactive sensory neuropeptide, CGRP released with its activation, in the pathomechanism of fibrotic and inflammatory skin mutation induced by bleomycin.

2. a) Second phase of the examinations was aimed at discovering the role of capsaicin-sensitive afferents and the somatostatin of sensory origin in chronic arthritis and subsequent hyperalgesia in rheumatoid arthritis rat model.
b) In addition we also examined the effects of synthetic heptapeptide sst_4 receptor antagonist TT-232 in this same model.
c) Furthermore we also aimed at examining the activation/sensitization mechanisms of TRPV1 receptors localized on the sensory nerve fibers, in chronic arthritis knockout mice with the help of enzyme inhibitors.

CHAPTER I

THE ROLE OF THE TRPV1 RECEPTOR AND CGRP IN BLEOMYCIN INDUCED SCLERODERMA MOUSE MODEL USING RECEPTOR KNOCKOUT MICE.

1. Literary background to our experiments:

Scleroderma is an autoimmune disease of unknown origin, most frequently it appears in the forties, and is ten times more common in women.

The characteristic features of the disease can affect only the skin and the subcutaneous tissues, in this case we are facing local scleroderma, or it can affect various internal organs as well, in which case it is called systemic. Systemic can be divided into diffuse and limited forms.

The increased collagen biosynthesis of fibroblasts has already been proved to contribute to the development of the condition, however, it is still unknown, what induces the cells to figuratively 'strangle' the tissues with collagen. Two main hypotheses are known regarding the etiology. According to the immunological hypothesis, fibrosis is a secondary anomaly, which is due to the abnormal activation of the immune system. It has been presumed, that the T-cells responding to the unknown antigen accumulate in the skin and effectuate the fibrotic alterations through the cytokines produced by them. By the vascular hypothesis, recurrent lesions of the micro vascular endothelium are the primary etiology factors.

The areas affected by the disease are the skin, the muscular-skeletal system, the gastrointestinal tract, the lungs, the kidneys and the heart. Currently there is no medicine that can cure scleroderma, treatment is primarily symptomatic.

Scleroderma, similarly to other autoimmune diseases, most frequently commences with Raynaud-symptom, which means the symmetrical pallor and cyanosis of the acral areas of the body due to cold or emotional stress factors. It is already a known fact, that in the skin samples of scleroderma patients showing signs of Raynaud manifestation, the number of CGRP immunoreactive neurons is significantly reduced compared to healthy control patients.

Several animal models have been designed for the experimental study of scleroderma but hitherto there is no model that carries all the features of clinical scleroderma.

We used a belomycin induced local scleroderma model designed by Yamamoto for our experiments. In this model alteration typical of scleroderma developed as a result of continuous local bleomycin treatment.

Bleomycin is an antitumor antibiotic produced by Str. verticillus. It works well on planocellular carcinomas of different localization. It is used especially in the treatment of scrotum cancer, head-neck tumors, lung carcinoma and esophagus carcinoma. Fibrosis of the lungs is a well-known side-effect.

2. Materials and methods:

For 30 days 0,1 ml 100 μg/ml concentration of bleomycin was injected under the dorsal skin of mice, the control group got the solvent of bleomycin, phosphate buffer (PBS) in the same volume, under the dorsal skin. The animals were then divided into four-four groups.

During examination of the role of TRPV1 receptor bleomycin treated $TRPV1^{+/+}$ (wild), bleomycin treated $TRPV1^{-/-}$ (KO), PBS treated $TRPV1^{+/+}$ (wild) and PBS treated $TRPV1^{-/-}$ (KO) groups were created, number of individuals in each group was 10-12 animals.

In the experiments aimed at the clarification of the role of CGRP receptor, bleomycin treated $CGRP^{+/+}$ (wild), bleomycin treated $CGRP^{-/-}$ (KO), PBS treated $CGRP^{+/+}$ (wild), PBS treated $CGRP^{-/-}$ (KO) groups were formed, with 8-10 individuals in each.

On the day following the administration of the last bleomycin injection, the mice were narcotized to sleep, and a section 6 mm in diameter was cut out from their nape. Samples were submitted to the following examinations:

Histological examinations:

After embedding in paraffin, the samples were stained with haematoxylin eosin and picrosyrius which paints collagen intent red. The degree of the evolving sclerosis was determined via semiquantitative histological scoring. In every sample, thickness of skin, intensity of the inflammation, structured form of collagen bundles were scored form 0-4, thus the final score could be in the range 0-12.

Measuring thickness of skin:

Thickness of cut-out skin was determined through 40x magnification, expressing the distance between dermo-epidermal junction and dermo-subcutan junction in micrometers.

Determining the number of myofibroblasts:

Determining the number of myofibroblasts proliferating in scleroderma-affected skin was done by immunohistochemical staining of α-smooth muscle actin (α-SMA) which is typical in myofibroblasts.

Determining hydroxiproline content biochemically:
Hydroxiproline is almost exclusively found in collagen of connective tissue and in elastin, therefore it is widely used as means of detecting the presence and amount of collagen. Hydroxiprolin content of the samples was determined using spectrofotometric methods, by a previously prepared standard curve. Values were expressed in microgram hydroxiproline content.

Quantitative analysis of the level of type-I collagen α1-chain mRNA, by RT-PCR method:
Total RNA content of the samples were isolated, the examined specific mRNA levels were amplified and determined by using LightCycler quantitative PCR system.

Statistical methods:
Results were expressed in mean ± SEM, applied to each group. For analysing the experimental methods, non-parametric Mann Whitney U-probe was used. A p-value ander 0.05 was considered significant difference among the analysed groups.

3. Results:

3.1. Examining bleomycin induced model in $TRPV1^{+/+}$ (wild type) mice:
The cumulative sclerosis score was 58% higher (6.33±0.19 and 4.00±0.31), skin thickness was increased by 42% (393.05±15.41 µm and 278.62±11.38 µm), and number of α-SMA-positive cells was extended by 75% (16.33±3.31 and 9.3±1.34 in each focus) in bleomycin treated $TRPV1^{+/+}$ group, compared to the PBS treated $TRPV1^{+/+}$ group.
Similarly to the histological alterations, the content of spectrofotometrically determined collagen specific amino acid, hydroxiproline was also significantly, by 47.5% higher (118.5±6.7 and 80.3±10.2 µg/skin sample) in the bleomycin treated group compared to the PBS-treated group of mice.
Treating with PBS in itself did not result in significant histological alterations as compared to skin samples of the untreated $TRPV1^{+/+}$ mice. There was no detectable difference between untreated $TRPV1^{+/+}$ and $TRPV1^{-/-}$ mice's histological skin structure.

3.2. Examining the role of TRPV1 receptor in skin sclerosis:

As a result of bleomycin treatment histological alterations of $TRPV1^{-/-}$ (KO) animals were more expressed, the summarized sclerosis score was significantly higher (7.46±0.20 and 6.33±0.19) compared to the bleomycin treated $TRPV1^{+/+}$ (wild) group.

Value for thickness of the skin was also significantly higher (464.86±10.15 µm and 393.05±15.41 µm) and number of α-SMA-positive cells was also highly increased (24.03±3.07 and 16.33±3.31 in each focus) in bleomycin treated $TRPV1^{-/-}$ group compared to the also bleomycin treated $TRPV1^{+/+}$ group.

In accordance with the histological changes, the hydroxiproline content of the samples was also 57% higher (186.605±8.4 and 118.5±6.7 µg/skin sample) in bleomycin treated $TRPV1^{-/-}$ group compared to $TRPV1^{+/+}$ group. There was no sognificant difference neither with histological methods or biochemical measuring of hydroxiproline content between PBS treates $TRPV1^{-/-}$ and PBS treated $TRPV1^{+/+}$ samples.

3.3. Analysing the expression of type-I collagen α1 chain mRNA in sclerotic skin:

Examining the levels of type-I collagen α1 chain mRNA, compared to the wild, PBS treated group, no significant difference can be detected in mRNA expression of the groups. Significant difference could not be found in levels of type-I collagen α1 chain mRNA by the end of the 4-week treatment, hence the degree of type-I collagen α1 chain mRNA in animals killed in an earlier phase of the experiment (after 2 weeks) was also examined, but significant discrepancy could not be detected resulting from bleomycin treatment.

Based on the results, it can be concluded that bleomycin treatment does not affect expression of type-I collagen α1 chain mRNA.

3.4. Examinig the role of CGRP receptor in scleroderma model:

Histological examinations showed that the complex sclerosis score was 42% higher in bleomycin treated $CGRP^{+/+}$ group (4.25±0.38 and 2.98±0.51), 52% higher skin thickness values (434.49±16.41 µm and 285.85±17.36 µm) and 62% more α-SMA-positive cells (15.83±3.6 and 9.75±0.88 in each focus) compared to the respective PBS treated groups. The amount of hydroxiproline increased by 47% (137.6±14.58 and 93.4±10.65 µg/skin sample) as a result of bleomycin treatment compared to control $CGRP^{+/+}$ PBS treatment.

Absence of CGRP receptor resulted in increased sclerotic changes with bleomycin tretment, the complex sclerotic score was significantly increased (6.21±0.58 and 4.25±0.47) in

bleomycin treated $CGRP^{-/-}$ (KO) group compared to bleomycin treated $CGRP^{+/+}$ (wild) group. Skin thickness showed an increase of 29% (557.23±18.38 µm and 434.49±16.41 µm) in bleomycin treated $CGRP^{-/-}$ group as compared with the similarly treated $CGRP^{+/+}$ group. The number of α-SMA-positive cells also increased significantly (27.83±4.58 and15.83±3.60 in each focus) in bleomycin treated $CGRP^{-/-}$ group compared to the also bleomycin treated $CGRP^{+/+}$ group.

In accordance with the histological changes, hydroxiproline content of the samples was also significantly higher (179.30±18.90 and 137.60±14.58 µg/skin sample) in bleomycin treated $CGRP^{-/-}$ group compared to $CGRP^{+/+}$ group.

Surprisingly, on the skin of PBS treated $CGRP^{-/-}$ animals increased sclerotic changes were also perceivable as an elevated complex sclerosis score (3.96±0.49 and 2.98±0.51), higher skin thickness values (386.25±10.26 µm and 285.85±17.36 µm), increased α-SMA-positive cell count (15.04±2.39 and 9.75±0.88 in each focus) and increased hydroxiproline content (126.56±13.10 and 93.4±10.65 µg/skin sample) could be observed compared to the PBS treated $CGRP^{+/+}$ group. The sclerotic changes developing in PBS treated $CGRP^{-/-}$ group were similar in extent to those in the bleomycin treated $CGRP^{+/+}$ group, therefore we examined the non-treated CGRP knockout and wild mice skin samples as well and we discovered that there is no significant difference neither in histological structure, nor in hydroxiproline content between non-treated animals.

4. Discussion, conclusion:

In these series of experiments we focused our study on the role of TRPV1 receptor and CGRP in chronic fibrotic-sclerotic circumstances. The 30-day bleomycin treatment in the $TRPV1^{+/+}$ mice created from C57BL/6 tribe, resulted in approximately 150% skin thickening, remarkable, histological estimated skin sclerosis, 75% more increased α-SMA-positive cell count, and 148,5% increased hydroxiproline content as compared to the PBS treated $TRPV1^{+/+}$ group used as control. These results were in accordance with previous results in literature regarding the C57BL/6 tribe. In contrast with previous findings the increase in the level of type-I collagen α1 chain mRNA could not be detected via quantitative PCR. It is not accurately clarified what might be the background of this observation, however the elevated amount of collagen protein beside normal mRNA levels can be deduced to a number of reasons, e.g.: a decrease in the degradation of collagen, increased translation, increased collagen maturing processes.

Absence of TRPV1 receptor in the examined bleomycin induces scleroderma model definitely aggravates sclerotic skin alterations, supported by histological and biochemical examination results. In bleomycin treated $TRPV1^{-/-}$ (KO) animals there was a significantly higher complex sclerotic score, higher skin thickness values, an elevated number of α-SMA-positive cells and az increased hydroxiproline content was measured as compared with the similarly bleomycin treated $TRPV1^{+/+}$ (wild) animals. There is no detectable difference between the skin structure of non-treated knokcout and wild animals, therefore the absence of TRPV1 receptor in itself does not cause fibrotic-sclerotic changes. For sclerotic alterations to develop profibrotic factors, like bleomycin are needed to be present.

Based on our results it can be declared, that presence of the TRPV1 receptor has a protective effect against sclerotic alterations induced by profibrotic agents. Supposedly the inflammatory mediators from inflammatory processes induced by bleomycin activate and sensibilize the TRPV1 receptor, which leads to the emission of sensory neuropeptides, which have protective effects against phatological changes, in the models studied in our research.

From the sensory neuropeptides released during activation of TRPV1 receptor data can only be found on CGRP in the literature, as a potentially protective factor in the pathomechanism of scleroderma and Raynaud's phenomenon. Based on these observations we used CGRP knockout mice to clarify the role of CGRP in sclerotic processes in the in vivo bleomycin induced scleroderma model used in our study. In $CGRP^{+/+}$ group, created from C57BL/6 tribe, bleomycin treatment resulted in relevant morphological, biochemical, histological and immunohistochemical (α-SMA) changes. In the absence of CGRP, bleomycin treatment definitely caused increased sclerosis, regarding skin thickness, histological score, hydroxiproline and α-SMA. Our experiments provide the first in vivo proof of sclerotic alterations developing in the absence of CGRP. Presumably the vasospasm and resulting ischemia is in the background, caused by the lack of CGRP's vasodilatative effects.

Concluding the results of our study, we can state, that presence of TRPV1 receptor and CGRP proves to have a definite protective role against the pathomechanism of scleroderma. CGRP agonists can have future therapeutic potential in the treatment of scleroderma, but naturally, the possible side-effects will have to be taken into consideration as well.

CHAPTER II.

THE ROLE OF CAPSAICIN SENSITIVE NEURON ENDINGS AND TRPV1 RECEPTOR IN ADJUVANT-INDUCED CHRONIC ARTHRITIS MODEL

1. Literary background to our experiments:

Rheumatoid arthritis primarily affects young, middle-aged women, in Hungary some 70-80 thousand patients can be taken into account. This disease is a chronic, progressive multi-joint inflammation of autoimmune origin, which causes pain, a disabled state, disability and a serious deterioration in the quality of life via destruction of joints and deformities. The joint case and the synovium are richly innervated with capsaicin-sensitive fibers. These nerve endings mediate strain and pain, furthermore numerous literary data justifies that the neurogen inflammation mediated by them plays an important role in the development of chronic arthritis. In the synovial fluid of patients suffering from arthritis, elevated levels of inflammatory sensory neuropeptides were shown. There is literary data however, proving that other neuropeptides (opiois peptides, galanin, somatostatin) also released from activated sensory nerve endings inhibit the development of chronic arthritis in experimental circumstances. Concerning arthritis, there is literary evidence for the pathophysiological relevance of both inflammatory and anti-inflammatory neuropeptides released from capsaicin-sensitive neuron endings.

Current therapy of chronic arthritis is primarily only eases the symptoms. At first non-steroid anti-inflammatory drugs/painkillers are recommended, while in an advanced state of the disease steroids and later immunosuppressive base-therapy drugs (methotrexate, leflunomid) can be administered. The causal therapy of this group of diseases is thus far from being solved, and there is no single drug on the market which could effectively block the neurogenic component of inflammation.

2. Materials and methods:

Chronic arthritis was triggered by complete Freund adjuvant (CFA, killed Mycobacterium's suspension in paraffin oil, 1mg/ml), in rats, 100 microliters, in mice 50 microliters subcutaneously into the left soles and to the tail stem. Inflammatory reaction develops in the tibio-tarsal joints, accompanied by systemic inflammatory symptoms (elevation in body temperature, weight loss, fatigue) which are more pronounced on the side of the injection, as a result of CFA, but can nonetheless be observed on the opposite side as well.

A. Experiments with rats:

In our experiments, 4 animal groups were formed, and in each of them, arthritis was induced as described above.

To examine the role of capsaicin-sensitive afferent sensory fibers, one group of rats were pre-treated by RTX, a TRPV1 antagonist, which causes damage to these sensory nerve endings. During the pre-treatment, on three consecutive days 30, 70 ans 100 μg/kg RTX was given to the animals subcutaneously under the skin of the neck. Arthritis was induced 7 days later. In order to verify the success of RTX desensibilisation, before starting the experiment, wiping test was performed by dropping capsaicin into the eyes (50 μl of 0,1% capsaicin), the avoiding wiping reaction was absent in all pre-treated animals.

For the study on the role of somatostatin – which is released when capsaicin sensitive nerve endings are activated – another group of the rats was treated with sst receptor antagonist cyclo-somatostatin (C-SOM) daily, for 21 days (20 μg/kg i.p.).

In the next series of experiments, the rats were treated with synthetic heptapeptide sst_4/sst_1 agonist TT-232 during the entire procedure of the experiment (2x50-400 μg/kg/nap i.p.), in the control group, solvent was used.

B. Experiments with mice:

To study the possible activation of TRPV1 receptor in chronic arthritis' conditions, a group of both TRPV1 receptor knockout and wild-type animals (n=8-12/group) were treated by one of the following materials from the beginning of the adjuvant therapy, every day, during the entire experimental procedure. The tested compounds were chosen to identify the possible mediators playing a role in the activation of the TRPV1 receptor.

To study the role of lipooxygenase enzyme products in the activation of TRPV1, one group of animals was treated with nor-dihydro-guaretic acid (NDGA), a non-selective lipoogygenase-inhibitor, in a dose of 25 mg/kg/day intraperitoneally (ip.). The second group of animals was treated with bradykinin B1 receptor antagonist desArgHOE-140 to reveal the possible sensitizing effect of bradykinin, while the third group was treated with bradykinin B2 receptor antagonist HOE-140 (in both cases 250 μg/kg/day ip.).

Mice of the fourth group got indomethacin (1mg/kg ip.), a non-selective cyclooxygenase enzyme inhibitor, to study the possible TRPV1 receptor activating and sensitizing effect of cyclooxygenase enzyme products.

As control group for the individual group's studies, there was a group treated with i.p. saline.

Test methods:

Paw edema measurement:

Volumes of the legs were measured with Ugo Basile 7140 plethysmometer, which is a device operating on the principle of communicating vessels. The volumes were read off from the digital display in cm^3-s on the day 2, 5, 8, 12, 15, 18, 21 of the experiment, the measured data were then compared with data from the baselin and expressed as a percentage of the edema.

Measurement of the mechanonociceptive treshold:

Ugo Basil dynamic plantar esthesiomether was used to determine the mechanociceptive threshold of the feet. This is the digitalized version of classical von Frey method.

During the experiment, the middle region of the animals' feet was stimulated with a stainless steel flat mandrel, with pre-set dynamic parameters. The threshold value at which the animal lifts its feet can be read from the digital display expressed in grams.

Every result was calculated from the average of three consecutive measurements, the mechanical threshold was expressed compared with the initial values, in percentages.

Preparation and evaluation of histological sections:

Tibiotarsal joint of narcotized animals, killed by cervical dislocation was cut out, was fixated in formalin and then it was decalcinated. When the joints softened enough, samples were embedded into paraffin, then cut into 5-7 μm slices and then stained with hematoxylin-eosin.

The inflammatory changes were then evaluated by an independent, experienced pathologist colleague. Semi quantitative scoring was based on the scale of thickening of the synovial connective tissue, leukocyte infiltration, and on the rate cartilage and bone damage separately. Each of the four parameters were scored on a scale from 0 to 3, the points were added, thus for every sample, and thus for every group a complex arthritis score was obtained with a value ranging from 0 to 12.

Determination of plasma somatostatin concentration:

At the end of the experiment, 4 ml blood samples were taken from each rat via punctuating the heart in deep anesthesia. The blood samples were transferred into iced tubes containing EDTA and aprotonin. After centrifugation the plasma peptide content was extracted with

absolute alkohol, the supernatant was dried under a stream of nitrogen, and was then resolved in RIA-buffer. Somatostatin-like immunoreactivity of the plasma was determined by RIA.

Statistical analysis:

To statistically analyse of paw edema, allodynia/hyperalgesia after two-way ANOVA, Bonferroni correction t-test was used. For analyzing the complex arthritis score and plasma peptide concentrations, the non-parametric Mann-Whitney U test was applied. A value of p under 0.05 was regarded significant difference in all cases in the studied groups.

3. Results:

3.1. The role of capsaicin sensitive fibers and the somatostatin released from them in a rat model of chronic arthritis:

In control non pre-treated animals, the foot treated with CFA gradually swelled, and on the eights day it reached 60% of the maximum, the volume increased from 0.73±0.02 cm^3 to 1.18±0.13 cm^3. On the contra lateral foot, only a smaller edema was observed, of 16-18%.

After inactivation of capsaicin-sensitive afferent fibers with RTX pre-treatment, the edema of the entire 21-day experimental period was larger, the daily applied C-SOM injection, which inhibits the effects of somatostatin on all five sst receptors, increased the foot swelling to the similar extent as the RTX pre-treatment.

In the control animals the CFA treatment caused the mechanociceptive threshold to decrease significantly, on the leg treated with CFA, it decreased by 25-30%, while on the contra lateral leg, by only 5-7%. The pre-treatment with RTX and C-SOM treatment significantly increased allodynia on both legs during the entire 21-day experimental period.

The histological sections show, that the CFA-treated left joint of the rats is damaged by the continually growing synovial pannus. The synovial cavity is expanded, synovial connective tissue is densely infiltrated with mononuclear cells, the cartilage is heavily eroded, and massive bone loss can be observed. Minor inflammatory changes can be seen in the contra lateral joints, cartilage and bone damage rarely occurs, mononuclear cell infiltration is significantly less severe, in some parts, the synovial tissue is thickened.

In the tibiotarsal joint of rats pre-treated with RTX and treated with C-SOM, as a result of CFA a much more pronounced synovial hyperplasia, more prominent thickening and mononuclear cell infiltration, and furthermore much greater cartilage damage and advanced bone loss can be seen. In both groups, the contra lateral joints show more intense

inflammatory lesions. The arthritis score results have also confirmed that both RTX pre-treatment, and C-SOM treatment significantly aggravate the arthritis.

SOM-like immunoreactivity of the plasma increased steadily following the CFA-injection, and by the 21st day, it reached four times the maximum value. Pre-treatment with RTX significantly reduced SOM release during the inflammation, the basal level did not change.

3.2. Effect of TT-232-treatment on CFA-induced chronic arthritis:

TT-232 decreased the CFA-induced leg-edema in a dose-dependent way, the highest dose causing 50% inhibition on the CFA-treated leg. Highest doses of TT-232 prevented the 30% mechanical allodynia measured in the control group, and moreover, surprisingly it has also resulted in a rise in the threshold value. Edema and hyperalgesia completely ceased on the contra lateral side. Tibiotarsal joint of the rats treated with the highest doses, showed signs of milder inflammatory reactions. Synovial swelling, lymphocyte accumulation and destruction of cartilage was reduced, bone damage did not develop. This is also confirmed by the complex arthritis score, which was significantly smaller on both legs as compared with the control group treated with solvent. During the entire experimental period the TT-232 injected intraperitoneally two times per day inhibited the CFA-induced elevation of somatostatin-like immunoreactivity of the plasma in a dose-dependent manner. The highest dose reduced the somatostatin concentration to the basal value.

3.3. CFA-induced paw edema, mechanical hyperalgesia and histological changes in TRPV1$^{+/+}$ and TRPV1$^{-/-}$ mice:

In wild mice, paw edema on the treated side increased steadily, reaching the maximal swelling on the 16th day (130% swelling compared to the baseline value), on the contra lateral leg, smaller edema developed (30%). In case of TRPV1$^{-/-}$ mice significantly smaller swelling developed on both sides from the first day of measurements, throughout the entire experimental period (a maximum of 80% swelling on the treated and 15% on the contra lateral leg).

Mechanociceptive threshold in the CFA-treated leg of wild-type animals decreased by 45-50%, while on the contra lateral side, only 10-15% hyperalgesia was developed. In contrast, threshold value of TRPV1$^{-/-}$ mice showed only 30-35% decrease on the treated leg, while no hyperalgesia developed on the contra lateral side.

Compared to intact joint structures, in histological sections of CFA-treated tibiotarsal joints of wild mice, tissue destruction could be found similar to those observed on rats. There was a

significantly smaller degree of cartilage destruction without bone damage in $TRPV1^{-/-}$ mice, and thickening of the synovial tissue and mononuclear cell infiltration was also somewhat milder, consequently the complex arthritis score was also significantly lower (7.8 ± 1.1 vs. 4.6 ± 1.2).

3.4.

3.4. Effect of desArgHOE-140, HOE-140, NDGA and indomethacin on CFA-induced paw edema, hyperalgesia and histological alterations in wild-type and $TRPV1^{-/-}$mice:

The bradykinin B_1 receptor antagonist desArgHOE-140 did not induce any significant alterations on either CFA-evoked edema or mechanical hyperalgesia and did not remarkably altered the composite arthritis score. In contrast, the bradykinin B_2 receptor antagonist HOE-140 exerted a 30-40% inhibition on paw swelling and hyperalgesia throughout the total duration of the study, the histological score was also reduced by 55%. The inhibitory action of the non-selective lipoxygenase inhibitor NDGA was 40-50%, while that of the cyclooxygenase inhibitor was 60-80% on the inflammatory mechanical hyperalgesia. NDGA diminished edema formation by 25-30%, indomethacin by 40-55%, and the arthritis score was also decreased by 40% and 65% in response to treatments with these agents, respectively.

In the $TRPV1^{-/-}$ group, however, NDGA and HOE-140 did not influence any of the inflammatory parameters. Treatment with the B1 receptor antagonist desArgHOE-140 was not investigated in the knockout group, since it did not have any statistically significant action in the wild-types. Indomethacin decreased hyperalgesia by 50-60%-kal and edema by 20-30%. In contrast to what was seen in the wild-type animals, indometacin did not decrease edema in $TRPV1^{-/-}$ mice in the early period of the experiment, the first significant action as observed 11 days after CFA injection. Furthermore, both the antihyperalgesic and the anti-edema effects were smaller than that found in the $TRPV1^{+/+}$ group. No effects of NDGA and HOE-140 was seen on the inflammatory histopathological parameters in $TRPV1^{-/-}$ mice compared to the solvent-treated control group, while indomethacin treatment resulted in 40%-decrease of the composite inflammation score.

4. Discussion and conclusions:

We provided direct chemical evidence in a rat model of chronic arthritis that stimulation of the peripheral terminals of capsaicin-sensitive primary sensory nerves results in a 4-fold increase of somatostatin level in the systemic circulation. This was markedly inhibited after the inactivation of the capsaicin-sensitive afferents by RTX pretreatment. In chronic arthritis a

variety of receptors and ion channels on these sensory nerves of the joints are presumably activated. As a result of all all these, SOM is released, gets into the systemic circulation and exerts anti-inflammatory and anti-hyperalgesic actions. These are concluded according to the fact that the non-selective sst receptor antagonist C-SOM-treatment made the inflammatory parameters more severe similarly to RTX-pretreatment. These results provide evidence for the protective role of capsaicin-sensitive fibres in chronic arthritis and endogenous counter-regulatory mechanism mediated by somatostatin of sensory neural origin. Based on all these data, SOM might have interesting for therapeutic purposes, but it cannot be an ideal drug candidate due to its 3-min plasma elimination half-life and its broad range of other, particularly endocrine, actions through the activation at all sst receptors. Stable, synthetic somatostatin agonists, particularly non-peptide ligands, which potently decrease inflammatory and nociceptive processes without influencing hormone secretion, might open promising perspectives for the development of a completely novel group of safe anti-inflammatory and analgesic drugs.

On the basis of these results, in the next series of experiments we investigated the effects of the selective sst_1/sst_4 receptor agonist TT-232, which does not exert any endocrine actions, in the same rat model of chronic arthritis. Chronic treatment with TT-232 (2x400 μg/kg i.p.) reduced paw edema by about 50% and turned hyperalgesia into hypoalgesia. Furthermore, the inflammatory histopathological parameters were also remarkably attenuated, only a small pannus formation, moderate inflammatory cell infiltration and minimal cartilage destruction were observed, but no signs of bone destruction could be detected.

In the second experimental setup, the adjuvat-induced chronic arthritis model originally developed in Lewis rats were adopted and modified for C57Bl/6 mice. We proved that this is a reliable model also in this strain, since a 130% edema and 50% mechanical hyperalgesia developed on the injection side.

Studies with $TRPV1^{-/-}$ mice in this CFA-induced arthritis model clearly revealed that the lack of this ion channel localized on the capsaicin-sensitive significantly decreased all inflammatory parameters on both side. Our results are the first evidence that the TRPV1 receptor plays an important regulatory role in chronic arthritis and consequent mechanical hyperalgesia. It can be assumed that pro-inflammatory sensory neuropeptides released in response to its activation aggravate the intensity of the inflammatory response in the joints and also contribute to the decrease of the mechanonociceptive threshold.

As a next step we aimed at identifying the inflammatory mediators which are responsible for the activation of the TRPV1 receptor. After an extensive literature search, the roles of

bradykinin, lipoxygenase and cyclooxygenase products were investigated. It is important to emphasize that we did not want to analyze which mediator is the most important in the development of the inflammatory reaction, but the difference observed in the effects of these agents in the wildtype and TRPV1$^{-/-}$ groups. The lipoxigenase enzyme inhibitor nor-dihydro-guaretic acid (NDGA) significantly reduced the mechanonociceptive threshold in the treated wild-type animals from the second experimental day. On the contrary, the antinociceptive and anti-inflammatory effects were completely missing in the TRPV1 KO animals. The daily treatment with bradykinin B2 antagonist HOE-140 reduced the development of edema formation and mechanical hyperalgesia in the wild-type group but these effects were not shown in KO mice. These observations support previously published in vitro data, that bradykinin released during inflammatory processes acting on bradykinin B2 receptors sensitizes TRPV1 receptors on the peripheral sensory nerve endings. The bradykinin B1 receptor antagonist desArgHOE-140 treatment did not influence significantly inflammatory changes in the wild-type group. On the basis of these results we conclude that bradykinin B1 receptors do not play role in the chronic joint inflammation model in mice. The cyclooxygenase enzyme inhibitor indomethacin significantly reduced (in the highest degree among the examined compounds) the arthritis and mechanical hyperalgesia in both treated animal groups compared to the solvent treated animals but the degree of inhibition was remarkably smaller in the TRPV1 receptor gene-deficient mice especially in the inicial phase of the chronic inflammation. According to the present experimental data, it is concluded that prostaglandins released from the inflamed tissue contribute to the enhancement of edema by activation/sensitization of TRPV1 receptors.

The lack of TRPV1 receptors on the capsaicin-sensitive sensory nerve endings, in contrast to the total inactivation of the nerve ending-does not increase but notably inhibited the signs of the arthtritis and hyperalgesia. This apparent contradiction can be explained with the facts that proinflammatory and anti-inflammatory neuropeptides are released by activation of different receptors-/ion channels. Activation of TRPV1 receptors mainly causes the release of proinflammatory neuropeptides. The genetic lack of TRPV1 receptors significantly inhibits the inflammation and hyperalgesia in mice. We presume other mechanisms in the release of somatostatin. Previous data have been published that sensory neuropeptides only partly co-localize in the capsaicin-sensitive afferents (Hökfelt és mtsai., 1976), it can be supposed that somatostatin-containing nerve endings express dominantly not TRPV1 but even more TRPA1, bradykinin- and purinergic, etc. receptors.

We have indentified some potential target molecules, the TRPV1 ion channel and somatostatin sst_4/sst_1 receptors. Selective TRPV1 antagonists, or sst_4/sst_1 agonists can be new perspectives in the pharmacotherapy of the chronic arthritis.

SUMMARY OF ORIGINAL FINDINGS OF THE THESIS

1. On the basis of our experimental data we can conclude that TRPV1 receptors and CGRP have protective role in the pathomechanism of scleroderma. CGRP receptor agonists can be new perspectives in e pharmacotherapy of scleroderma in the future but we have to consider the potential unwanted effects.

2. We have presented evidence that somatostatin released from the capsaicin-sensitive sensory nerve endings plays protective role in the development of arthritis.

3. A stable synthetic heptapeptide sst_4/sst_1 receptor agonist TT-232 efficiently inhibits the chronic joint inflammation (edema and histopathological changes) and inflammatory mechanical allodynia as well; therefore it can open promising perspectives in the drug development.

4. It has been proven that TRPV1 ion channels expressed on the capsaicin-sensitive afferents can be sensitized by bradykinin, prostaglandins and lipoxigenase products. Selective TRPV1 receptor activation, in contrast to the stimulation of the whole sensory nerve endings, increases the inflammation and allodynia. On the basis of these observations, the usage of selective TRPV1 receptor antagonists could also mean new therapeutic tools in the treatment of arthtritis

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