INVESTIGATION OF NOVEL ANTI-INFLAMMATORY AND ANALGESIC MECHANISMS IN CLINICAL STUDIES AND ANIMAL MODELS

Ph.D. thesis

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INTRODUCTION

Capsaicin-sensitive sensory nerves

The present thesis -similar to other Ph.D. works have made in our department in the past 25 years- is based on the results of investigations of capsaicin-sensitive sensory neurons by Prof. János Szolcsányi and Prof. Erika Pintér. The capsaicin-sensitive sensory neurons are small myelinated Aδ- and unmyelinated C-fibers. They can be activated by capsaicin (8-methyl-*N*-vanillyl-6-nonenamide, the pungent compound of pepper/*Capsicum anuum*) via the transient receptor potential vanilloid 1 (TRPV1) receptor (Caterina et al., 1997). These nerve terminals are special because they have three distinct functions. On the one hand they play a role in the nociception that is the afferent function. On the other hand these nerve endings have a local efferent function because their stimulation causes local neurogenic inflammation (vasodilatation and plasma protein extravasation leading to edema formation) via release of pro-inflammatory neuropeptides such as tachykinins (substance P/SP, neurokinin A/NKA), calcitonin gene-related peptide (CGRP) (Szolcsányi, 1984; 1988; Maggi and Meli, 1988; Helyes et al., 2003). Szolcsányi and Pintér have recognized that during local neurogenic inflammation evoked by antidromic electrical stimulation of spinal dorsal roots systemic anti-inflammatory and analgesic neuropeptides, such as SST also released from capsaicin-sensitive afferents. That is the third systemic efferent function of capsaicin-sensitive sensory nerve terminals ((Pintér and Szolcsányi, 1988; 1996; Szolcsányi et al., 1998a, 1998b; 2004; Helyes et al., 2000; 2004). It was discovered that low frequency (0.1 Hz) electrical stimulation of nociceptors caused maximal anti-inflammatory effect and elevation of plasma level of SST without any pain sensation (Szolcsányi et al., 2004). These findings have contributed to the development of novel anti-inflammatory and analgesic drugs which could act directly at somatostatin receptors. The capsaicin-sensitive afferents can also be activated via the transient receptor potential ankyrin 1 (TRPA1) ion channels which are coexpressed with TRPV1.

Transient Receptor Potential Vanilloid 1 (TRPV1) and Ankyrin 1(TRPA1) ion channels

The TRPV1 and TRPA1 receptors belong to transient receptor potential (TRP) receptor family. TRPV1 with other five ion channels is a member of TRPV subfamily and TRPA1 is the only member of TRPA subfamily. The molecular structure and distribution of TRPV1 and TRPA1 are similar and these ion channels are coexpressed on nerve terminals (Streng et al., 2008). TRPV1 and TRPA1 are non-selective ion channels, permeable to Ca^{2+} and Na^{+}. These ion channels are polymodal sensors. TRPV1 and TRPA1 cannot be classified as ligand-gated or voltage-gated ion channels. These receptors are molecular integrators of thermal, mechanical and chemical stimuli (Szolcsányi, 2008). The exogenous plant-derived vanilloid agents and endogenous antagonist of TRPV1 can stimulate the receptor at intracellular side (Jordt and Julius, 2002); protons can activate the receptor on the extracellular side (Jordt et al., 2000). TRPA1 agonists (e.g. H_2S) open the ion channel by covalent modification of cysteines in the N-terminal region of

the receptor (Hinman et al., 2006). Upon activation, Na^+ and Ca^{2+} pass through the channels' pore. Natrium ions generate action potentials and cause nociception, calcium ion influx leads to the release of sensory neuropeptides from nerve terminals. Permanent or repeated activation causes increase of intracellular Na^+ and Ca^{2+} concentration, which leads to dephosphorylation of the receptors, inhibits the voltage-gated potassium channels, generation of action potential and causes swelling of the mitochondria and cytoplasm. Therefore the nerve terminals become destroyed, desensitized (Koplas et al., 1997; Piper et al., 1999; Liu et al., 2001; Dedov et al., 2001; Szolcsányi, 2003).

Hydrogen-sulphide (H_2S) and the capsaicin-sensitive sensory nerves

Patacchini et al. (2004) have proved that H_2S activates the capsaicin-sensitive peptidergic sensory nerves leading to release of pro- and anti-inflammatory neuropeptides, therefore H_2S could have important role in inflammatory processes and neuro-immune interactions. In 2005 published data suggested that release of neuropeptides evoked by NaHS was reduced after desensitization of nerve endings or selective inhibition of TRPV1 (Trevisani et al., 2005). Later it was proved that NaHS has induced calcium response on TRPA1 receptor expressing on CHO cells (Streng et al., 2008). It has been also published that TRPA1 expressed on dorsal root ganglial cells has been activated by H_2S. The increased intracellular Ca^{2+} concentration could be inhibited by selective TRPA1 antagonists, however TRPA1 antagonists could not influence the response (Miyamoto et al., 2011). H_2S caused Ca^{2+} influx in sensory neurons, which could be inhibited by selective TRPA1 receptor antagonists. The H_2S -induced calcium response could not be detected in Trpa1 gene-deficient mice, however deletion of Trpv1 receptor did not influence the intracellular increase of Ca^{2+} (Ogawa et al., 2012). Therefore, it seems more likely that effects of H_2S are mediated by activating TRPA1 receptors.

The role of H_2S in inflammation and the molecular targets of H2S

H_2S is a small, colourless, diffusible gas which penetrates well through the skin and smells like rotten eggs. It is a toxic agent, however it is also known as an endogenous neuromodulator, inter- and intracellular signalling molecule in mammals besides nitric oxide (NO) and carbon monoxide (CO) (Li and Moore, 2008). In inflammatory processes H_2S exerts pro- and anti-inflammatory effects, depends on its applied concentration and the type of inflammation. H_2S scavenges reactive oxygen species, inhibits expression of endothelial and leukocyte adhesion molecules, release of TNF-α, chemotaxis and degranulation of neutrophil granulocytes and induces apoptosis of these leukocytes. Leukocyte adhesion effect of H_2S could be prevented by K_{ATP} channel inhibitor, glibenclamide pretreatment (Zanardo et al., 2006). H_2S can act on several different kinds of molecular targets which have been divided into four groups by Li et al. (2011). H_2S is able to modify certain kinds of proteins (sulfhydration) which can lead to enzyme activation and influences cell signalling pathways. H_2S affects the activity of kinases and

transcriptional factors. Through this mechanism, H_2S influences inflammatory processes and cell protection. H_2S has a metabolic effect, because this gas inhibits cytochrome c oxidase and ATP production. H_2S inhibits L-type calcium channels in cardiomyocytes and intracellular chloride channels in rat heart lysosomal vesicles; it activates K_{ATP} and TRPA1 channels.

Somatostatin: the anti-inflammatory and antinociceptive neuropeptide

Somatostatin exerts its inhibitory effects via five (sst1–5) G_i-protein-coupled receptors (Patel et al., 1995). The antinociceptive and anti-inflammatory actions of somatostatin are mediated by sst1, sst2 and sst4 receptors (Helyes et al., 2001; Pinter et al., 2002, 2006; Szolcsányi et al., 2004; Imhof et al., 2011; Shi et al., 2014). Somatostatin is synthesized and stored mainly in the TRPV1 and TRPA1 receptors expressing capsaicin-sensitive sensory neurons from the neuronal structures. It has been proved that somatostatin released from the activated peripheral terminals of capsaicin-sensitive sensory neurons exerts systemic anti-inflammatory and analgesic actions in acute and chronic models by our and other work groups (Szolcsányi et al., 1998a, b; Thán et al., 2000; Helyes et al., 2000, 2001, 2004; Pintér et al., 2002; Bar et al., 2004; Imhof et al., 2011). It has been established in animal experiments and pain models that exogenous SST and its analogues have anti-inflammatory and antinociceptive effect (Chrubasik, 1985; Karalis et al., 1994; Fioravanti et al., 1995; Lembeck et al., 1982; Szolcsányi et al., 1998b). The anti-inflammatory effect of SST is due to its inhibitory effect; SST inhibits the release of proinflammatory neuropeptides (e.g. SP) from nerve endings and their effects via sst receptors expressed on their target cells (ten Bokum et al., 2000). SST inhibits the release of other proinflammatory mediators, thus it has an immuno-regulatory function (Chowers et al., 2000; Elliott et al., 1999; Helyes et al., 1996, 2004; Szolcsányi et al., 1998a). SST reduces the IgA, IgM and IgE production of B lymphocytes, inhibits the IL-2, IL-4, IL-10 and interferon-γ (IFN-γ) release from T lymphocytes, chemotaxis of neutrophil granulocytes, activation of macrophages and natural killer cells (ten Bokum et al., 2000), and proliferation of lymphoid cells (van Hagen et al., 1994).

SST and its receptors are expressed in the pain processing and regulatory pathways in the central nervous system (dorsal horn of cervical and lumbar spinal cord; motor neurons in ventral horn of spinal cord; spinal cord dorsal root ganglion; hypothalamus; thalamus; trigeminal sensory nucleus; cortex; hippocampus; striatum) and in the periphery (Kumar, 2009). SST inhibits the nociception inhibition of central and peripheral neurons (Carlton et al., 2001; Helyes et al., 2000; 2004; Szolcsányi et al., 1998b). The molecular mechanism of pain relieving effect of SST is not well understood yet. Activation of G_i-protein-coupled SST receptors opens various potassium channels and inhibits voltage-gated calcium channels (Koch et al., 1988) resulting in inhibition of spike generation and release of neurotransmitters (Weckbecker et al., 2003), all of which can play a role in the antinociceptive effect.

AIMS

I. Several reports suggest the anti-inflammatory and analgesic effect of sulphurous medicinal waters in chronic inflammatory and degenerative diseases. There is little information in the literature about the molecular mechanism of action of thermal mineral waters. The present study aimed at investigating the effect of sulphurous medicinal water on psoriatic patients and in a murine dermatitis and osteoarthritis model and finding a possible explanation of the anti-inflammatory and analgesic mechanism. Furthermore, we planned to assess the role of TRPA1 receptor in mouse model of osteoarthritis.

II. Topical capsaicinoid treatments (creams, lotions and patches) with different concentrations of capsaicin (0.015–1%, 8%) have become widespread in inflammatory and arthritic conditions. Low-concentration capsicum patches have been described as an effective, analgesic, clinically relevant treatment in low back pain without systemic side effects. Most of the clinical studies investigate capsaicin or capsicum extracts in musculoskeletal pain conditions sometimes without knowing the active components of the applied product. Therefore we decided to perform qualitative and quantitative analysis of EMSPOMA®. The main aim of the present study was to evaluate the therapeutic potential of local capsaicinoid (EMSPOMA® cream) treatment on chronic low back pain in patients with degenerative spine diseases and to investigate the possible mechanism of action of the therapy.

III. The conventional thermonociceptive methods measure the latency (i.e. onset time) of nocifensive, pain-avoiding behavioural reactions evoked by a constant heat stimulus of suprathreshold intensity. These methods are not able to detect the actual noxious heat threshold. The study aimed at validating an increasing-temperature water bath suitable for determining the noxious heat threshold of mice, a species routinely employed in pain research. Validation included testing the effects of standard analgesics, neuroleptic and anxiolytic drugs in a parallel way on both the noxious heat threshold and the psychomotor activity assessed by the open field test to investigate whether psychomotor inhibition *per se* can lead to a thermal antinociceptive-like effect. Furthermore, assessing the interactions of either opioid or non-opioid analgesics with each other or with psychoactive drugs was also planned.

EXPERIMENTAL MODELS AND METHODS

I. INVESTIGATION OF EFFECT AND MECHANISM OF ACTION OF HARKÁNY SULPHUROUS MEDICINAL WATER IN PSORIATIC PATIENTS AND IN ANIMAL MODEL OF ALLERGIC CONTACT DERMATITIS AND OSTEOARTHRITIS; THE ROLE OF TRPA1 RECEPTOR IN ANIMAL MODEL OF OSTEOARTHRITIS

Clinical study

Nineteen patients (10 female, 9 male, 50–69 years) suffering from mild and moderate plaque psoriasis were involved in the study. Fourteen of them received 2×25 min bath in 36 ^{o}C Harkány medicinal water. Topical 0.1% dithranol ointment for 15 min daily was used as gold standard treatment during the 21-day study. Dithranol and bath treatment in tap water were applied in the control group (5 patients). Severity of the skin symptoms was characterized by Psoriasis Area and Severity Index (*PASI*, Smith et al., 2009). Before and after the balneotherapy 4 mm biopsy was taken from the marginal part of a psoriatic area. The excised regions did not receive topical dithranol treatment. The skin samples were fixed in 4% paraformaldehyde. Histopathological changes were determined on the hematoxylin–eosin stained sections. The amount and distribution of antigen presenting Langerhans cells were shown by immunohistochemistry using CD1a antibody (Dako, Denmark). Blood sample (10ml) was collected on the first and the last day before bathing to determine the level of SST-IR by a specific and sensitive radioimmunoassay (RIA) technique developed at our department (Németh et al., 1996). Patients were fasting before the blood withdrawal. In the analysis of SST plasma levels further 7 healthy volunteers were also included as controls (4 women, 3 men, 25–46 years). Data were presented in fmol/ml.

Animal models

We performed experiments on female, 20–25 g BALB/c mice in the dermatitis model, CD1, Trpa1 gene-deficient (KO, -/-) and wild type (WT, +/+) mice in osteoarthritis model. Mice were bred in the Animal Centre of the University of Pécs under standard pathogen-free conditions and maintained on standard diet and water *ad libitum* in climatically controlled environment.

1. Investigation of the effect of Harkány medicinal water in mouse model of allergic contact dermatitis

ACD was induced by oxazolone on the basis of our previous study (Bánvölgyi et al., 2005) adapted to the mouse paw. Mice were bathed in 37 ^{o}C medicinal water, tap water or distilled water (control groups) for 20 min/day. Paw volume was measured with plethysmometry (Ugo Basile, Italy) before and 4, 8, 24, 48 h after the induction of inflammation. Data were expressed in % of the initial values. Neutrophil accumulation was determined by assessment of myeloperoxidase activity, TNF-α concentration by ELISA (R&D Systems) from the dissected and homogenised paw skin samples. Semiquantitative scoring system was used to analyse histopathological changes on the haematoxylin–eosin stained sections.

2. Investigation of the effect of Harkány medicinal water and the role of TRPA1 in mouse model osteoarthritis (OA)

OA was induced in the left knee joint of mice by 20 μl, 25 mg/ml monosodium-iodoacetate (MIA, Sigma-Aldrich) injection (van der Kraan et al., 1989; Harvey and Dickenson, 2009). Mice were bathed in 37 ^{o}C medicinal water or distilled water (control groups) for 20 min/day. The volume of the knee joints was detected by digital micrometer (Mitutoyo, Japan) 3, 6 h after MIA injection and on the 2nd, 3rd, 4th, 7th, 9th, 11th, 15th and 21th days of the study. The size of the knee was shown on the digital screen in mm. The mechanical touch sensitivity of the paws was measured by dynamic plantar aesthesiometer (Ugo Basile, Italy) before the MIA injection and on the 2nd, 3rd, 4th, 7th, 9th, 11th, 15th and 21th days of the study. Data were expressed in percentage of the initial control values. Spontaneous weight bearing on the two hind limbs was determined by incapacitance tester (Linton Instrumentation, Norfolk, England) before the experiment and on the 2nd, 3rd, 4th, 7th, 9th, 11th, 15th and 21th days of the study. The percent weight distributed onto the MIA-treated hind limb was calculated by the following equation: [weight on the treated hind limb/(weight on the non-treated + weight on the treated)] x 100.

On the 22th day of the experiment knee joints were dissected and cross-sections (4 μm) were cut of the samples after paraffin embedding and stained with hematoxylin-eosin. Arthritic changes were scored using semiquantitative scoring system to characterise the MIA-induced histological changes (degree of the erosion and necrosis of the articular cartilage layer and changes in the synovium). Mean scores were determined from the sections of different animals, and composite score values were calculated from these mean scores.

3. Measurement of plasma level of SST after bath treatment of NaHS solution by nano-high-performance liquid chromatograph in on-line conjunction to electrospray ionization quadruple time-of-flight mass spectrometry (nanoHPLC-ESI-Q-TOF-MS)

Mice were bathed in a freshly prepared, 37 ^{o}C sodium hydrosulphide (NaHS, Sigma–Aldrich) solution or distilled water for 2 weeks. Sulphur concentration of NaHS solution was equal to Harkány medicinal water (12.4mg/l). Blood samples were collected by cardiac puncture from fasted animals. Plasma level of SST was measured by nanoHPLC-ESI-Q-TOF-MS. This technique is able to detect SST from small volume with high reliability and precision.

II. INVESTIGATION OF THE EFFECT AND THE MECHANISM OF ACTION OF LOCAL CAPSAICINOID THERAPY IN CHRONIC LOW BACK PAIN

Determination of capsaicinoids in EMSPOMA® cream by high pressure liquid chromatography–tandem mass spectrometry (HPLC–MS/MS)

The qualitative and quantitative analyses of capsaicinoids in EMSPOMA® cream were performed by HPLC–MS/MS using an Agilent 6530 Accurate Mass Q-TOF LC/MS system in according to the validated extraction and chromatographic method developed by Kaale et al. (2002).

Clinical study

Twenty patients (10 female, 10 male, 47–75 years) with chronic low back pain of more than 12 weeks' duration were included in the study (seven persons with discopathia lumbalis, seven with spondylosis lumbalis and six with post-laminectomy syndrome). Patients were enrolled by the rheumatologist of the Zsigmondy Vilmos Harkány Medicinal Spa Hospital from the inpatients who met the inclusion and exclusion criteria. The patients were examined by the same rheumatologist. Patients received treatment with EMSPOMA® cream containing 0.01% nonivamide (Jutta, Czech Republic).We applied the cream on a 25 × 40 cm area of the lumbar region of the spine (1 μg nonivamide/cm^2) as a pack for 30 min/day during the 21 day therapy. To assess the effect of pain on everyday life Oswestry Disability Index (ODI) was done on the first and the last day of the therapy. Data were expressed in percentage of the total score. A 100 mm visual analog scale (VAS) was used on the 1st, 7th, 14th, 21st days to record the low back pain as follows (Kulisch et al., 2009): VAS I: the severity of low back pain at rest, as rated by the patient; VAS II: the severity of low back pain upon exertion, as rated by the patient; VAS III: the perceived status, as rated by the patient; VAS IV: patient's progress, as rated by the investigator. Data were expressed in millimeters. To characterize the forward and lateral flexion of the lumbar region Schober's and Domjan's L and R tests (Domján et al., 1990) were performed on the 1st, 7th, 14th, 21st days. Data were expressed in centimeters. The plasma level of SST-like immunoreactivity was measured by radioimmunoassay (RIA) (Nemeth et al., 1996) on the first and the last day of the therapy before and after the nonivamide treatment. Data were presented in fmol/ml.

III. EFFECTS OF REFERENCE ANALGESICS AND PSYCHOACTIVE DRUGS ON THE NOXIOUS HEAT THRESHOLD OF MICE MEASURED BY AN INCREASING-TEMPERATURE WATER BATH

Female CD1 mice (25–35 g) were bred in the Laboratory Animal Centre of the University of Pécs under standard pathogen-free conditions and maintained on standard diet and water *ad libitum* in a climatically controlled environment. An increasing temperature water bath (Experimetria Kft., Budapest) was used to determine the noxious heat threshold of the mouse tail. Mice were gently held in an upright position above the water bath, and the tail was immersed into the water. The heating process was started with an initial temperature of 40°C and a heating rate of 24°C/min. The cut-off temperature was set to 53°C. The noxious heat threshold was defined as the temperature at which the animal withdrew or shook its tail. Subsequently, half of the group of animals was treated with the drug investigated (morphine, diclofenac, metamizol, diazepam, droperidol) and the other half with the same volume of the solvent (physiological saline) by intraperitoneal injection (0.1 ml/10 g). Heat threshold was determined 30 min. later, and the effect of each drug was assessed by comparison of the pre- and post-drug threshold values. The effects of drugs on spontaneous locomotor activity and exploratory behaviour were assessed by the open field test, which was performed 60 min. after drug administration on the same animals which were involved in the heat threshold

measurements. Locomotion (number of floor units entered and time of locomotion) and number of rearings were measured manually. The effect of each drug was examined by comparison with saline control by calculating percentage inhibition value for each activity measured in the open field test using the following formula: 100–(parameter measured in drug-treated animals/parameter measured in solvent-treated animals).

Statistics

Results are expressed as mean±SEM. Significant differences of PASI scores before and after the treatment in the group were defined by nonparametric Wilcoxon-test, between groups by unpaired t-test. Comparisons between control groups (distilled or tap water-treated) and medicinal water-treated animals or between wild type and knock out animals were made by ANOVA followed by Dunnett's post-test for edema, mechanonociceptive threshold, spontaneous weight distribution. Significant differences of neutrophil accumulation studies, cytokine and SST-IR data before and after the treatment in the group were determined by paired t-test, between groups by unpaired t-test. In the histological study nonparametric Mann-Whitney test was used. Significant differences of SST level in mice were determined by unpaired t-test between distilled water and NaHS treated group. Data were compared with Student's t-test in case ODI, Schober's and Domján's test. Friedman test followed by Dunn's multiple comparison test was applied for comparing VAS results, while Kruskal–Wallis test followed by Dunn's multiple comparison test was used for analysing plasma SST-LI levels. For comparison of the pre- and post-dose heat threshold values, the Wilcoxon matched-pairs test was used. For comparison of the psychomotor activity in the drug- and saline-treated groups, Student's t-test for unpaired samples was used. Statistical analysis was performed by the GraphPad Prism 5.1 software. Probability values *$p<0.05$, **$p<0.01$ and ***$p<0.001$ were accepted as significant.

Ethics statements

In the clinical studies all subjects were informed about the purpose, conditions and course of the study prior to inclusion. Patients have read and understood the Patient Information Sheet and have signed the Informed Consent Form. The study was approved by the Regional Research Ethics Committee and Ethics Committee of the University of Pécs (Approval No: 3787.316–5960/KK4/2010). The animal experiments complied with the ethical guidelines of the International Association for the Study of Pain (Zimmermann, 1983) and were performed according to the 1998/XXVIII Act of the Hungarian Parliament on Animal Protection and Consideration Decree of Scientific Procedures of Animal Experiments (243/1988). The studies were approved by the Ethics Committee on Animal Research of the University of Pécs (license No: BA 02/2000-11-2006; BA 02/2000-2/2012).

RESULTS AND DISCUSSION

I. INVESTIGATION OF EFFECT AND MECHANISM OF ACTION OF HARKÁNY SULPHUROUS MEDICINAL WATER IN PSORIATIC PATIENTS AND IN ANIMAL MODELS OF ALLERGIC CONTACT DERMATITIS AND OSTEOARTHRITIS; THE ROLE OF TRPA1 RECEPTOR IN THE ANIMAL MODEL OF OSTEOARTHRITIS

Clinical study

The mean PASI score did not change in the control group during the 21-day-long tap water bath and topical dithranol treatment. The mean PASI score was 7.74±1.60 on the 1st day and it was significantly reduced to 3.81±1.02 after the three week long Harkány medicinal water bath and topical dithranol treatment. In healthy volunteers basal plasma level of SST-IR was 10.07±0.61 fmol/ml. In the psoriatic patients it was significantly elevated (16.89±1.31 fmol/ml). At the end of the medicinal water bathing treatment plasma level of SST-IR increased significantly to 21.26±2.09 fmol/ml, but it did not change in the control group. In skin biopsy samples of the psoriatic patients thickened epidermis, leukocyte infiltration and dermal population of antigen-presenting Langerhans cells were recognised prior to the treatment with the medicinal water. The occurrence of dermal colonies of the dendritic cells represents the severity of the inflammatory changes in the skin. Balneotherapy reduced the amount of accumulated leukocytes and Langerhans cells in the dermis.

Animal models

1. Effect of Harkány medicinal water in mouse model of allergic contact dermatitis

Oxazolone caused robust paw edema. There were no significant differences between the distilled or tap water treatments applied in the control experiments. Swelling was significantly reduced (20.4±3.07-25.26±2.81%) by Harkány medicinal water. The level of MPO was significantly higher in the skin smeared with oxazolone. The bath treatment with medicinal water did not reduce the elevated level of MPO. TNF-α level in the skin samples was not changed by sulphurous medicinal water. According to the semiquantitative scoring Harkány medicinal water did not influence the histopathological changes.

2.a Effect of Harkány medicinal water in mouse model osteoarthritis

The bath treatment did not influence the MIA induced remarkable swelling of the knee joint. Although reduced hyperalgesia was measured in the medicinal water treated group, the difference was not significant. Significantly less decrease of weight distribution was observed in the medicinal water treated group from the 2nd to the 9th days compared to the control group. In the medicinal water treated group significantly milder histopathological changes were detected according to the semiquantitative scoring because of the moderate disorganization of the cartilage layer, synovial hyperplasia and reduced inflammation.

2.b Role of TRPA1 in mouse model osteoarthritis

The volume of the knee joint was significantly reduced in KO group 3 h after MIA-injection. However reduced hyperalgesia was measured in the KO group from the 3rd to the 11th day, the

difference between the two groups was significant on the 4th and 9th days of the study. Significantly less decrease of weight distribution was observed in the KO group on the 3rd, 7th and 11th days compared to the group of wide type animals. The histopathological score in the group of WT animals was 11.14±1.07 and 10.94±0.8 in the KO animals.

3. Changes of plasma level of SST after bath treatment of NaHS solution

Bath of NaHS solution significantly increased the plasma level of SST (29.13±1.758 fmol/ml) compared to distilled water treated group (24.12±0.58 fmol/ml) measured by nanoHPLC-ESI-Q-TOF MS.

The present study provided evidence for the molecular mechanism of anti-inflammatory and analgesic action of balneotherapy with sulphurous medicinal water in clinical observation and experimental animal models. Our results proved that H_2S content of the sulphurous medicinal water exerts significant increase of SST plasma concentration. Since SST is a well-known anti-inflammatory and analgesic neuropeptide which acts on the vascular phase of inflammation and modulates the function of inflammatory and immune cells and influence the nociception (Pintér et al., 2006), we presume that SST plays a role in the anti-inflammatory and analgesic mechanism of sulphurous medicinal water. The 37 ^{o}C distilled or tap water did not influence the inflammation, thus it can be excluded that the thermal effect of the water contributes to the anti-inflammatory action. Therefore, we suggest that the effectiveness of balneotherapy can be attributed to the chemical components of mineral waters. According to our data it is presumed that SST has a role in the improvement of psoriasis, allergic contact dermatitis and osteoarthritis by its inhibitory effect on the vascular and cellular phases of the inflammation and nociception. Furthermore, we proved that TRPA1 has role in the pathomechanism of MIA-induced osteoarthritis by transmission of nociceptive stimuli.

II. INVESTIGATION OF THE EFFECT AND THE MECHANISM OF ACTION OF LOCAL CAPSAICINOID THERAPY IN CHRONIC LOW BACK PAIN

Determination of capsaicinoids in EMSPOMA® cream by high pressure liquid chromatography–tandem mass spectrometry (HPLC–MS/MS)

Nonivamide was found as the only identified capsaicinoid molecule in the cream extract. The total amount of nonivamide within the extract of EMSPOMA cream is obtained 95.9 ppm ± 1.65% RSD (0.00959%).

Clinical study

We have found that ODI was significantly decreased on the 21st day from 39 ± 3.9% to 32.5 ± 4.4%.

All parameters which were characterized by VAS improved remarkably during the 3 week therapy like pain at rest (VAS I: −37.29%) and upon exertion (VAS II: −59.49%). There was a remarkable improvement in the perceived status of the patients (VAS III: −59.51%) and in

patient's progress (VAS IV: −59.44%) already during the first week of the therapy (VAS III: −29.71%, VAS IV: −16.71%). We could not find any significant changes in the flexion of the lumbar region as Schober's and Domján's tests showed. The plasma level of SST-IR showed threefold increase on the 1st day of the therapy after 30 min nonivamide treatment compared to baseline value (from 16.8 ± 3.12 fmol/ml to 56.9 ± 7.7 fmol/ml). On the last day of the study this significant difference could not be detected however the SST-IR before nonivamide treatment (19.5 ± 2.17 fmol/ml) was higher compared to healthy volunteers (10.07 ± 0.61 fmol/ml).

Topical capsaicinoid treatment has been used as analgesic in inflammatory and degenerative diseases since the nineteenth century; however the molecular mode of action has not been elucidated, yet. Analgesic effect of nonivamide has been proved in animal models (Skofitsch et al., 1984; Kawamura et al., 1993; Walpole et al., 1993). The present clinical study provided the first evidence that 21-day topical nonivamide therapy has analgesic effect in chronic low back pain and increases SST-IR in the human plasma on the 1st day. The anti-inflammatory and antinociceptive peptide SST is released from capsaicin-sensitive nerve endings and exerts systemic analgesic effect (Szolcsányi et al., 1998a, 1998b). There was no difference between the SST-LI levels before and after the topical nonivamide treatment at the end of the 21-day study, because repeated stimulation of the capsaicin-sensitive nerve endings causes depletion of the neural SST stores. Up to now it has been believed in the clinical practice that high concentration of the TRPV1 agonist capsaicin (8% Qutenza® patch) or repeated administration of lower concentration of capsaicin (0.015, 0.025, 0.075%) leads to desensitization of the capsaicin-sensitive nerve endings. Thus the nociceptor function of the nerve fibers and SP release – which is thought to be important signal for pain neurotransmission – also impaired for extended period (Bley, 2010; Kaale et al., 2002). These processes have been considered as a potential mechanism of analgesic action of topical capsaicin treatment, but several clinical studies proved that SP receptor antagonists have failed to be analgesics (Hill, 2000). Nonivamide is an agonist on TRPV1 receptors equipotent to capsaicin, according to the in vitro studies (Weiser et al., 2013). It is a more hydrophilic capsaicinoid (Tsai et al., 1994) associated with poorer partitioning ability into the skin (Fang et al., 1996). Antinociceptive effect developed in the deeper musculoskeletal and joint areas could not be explained by the desensitization of the cutaneous afferents (Anand and Bley, 2011). Endogenous SST released by nonivamide from the cutaneous afferents could reach own receptors expressed on articular neurons via the systemic circulation exerting analgesic effect by inhibition of their nociceptor excitability. Furthermore SST inhibits the release of nociceptive mediators such as proinflammatory neuropeptides (SP, CGRP), cytokines, prostaglandin and reactive oxygen species from inflammatory cells (Pinter et al., 2006). Besides the peripheral action SST exerts central analgesic effect (Spampinato et al., 1988).

III. EFFECTS OF REFERENCE ANALGESICS AND PSYCHOACTIVE DRUGS ON THE NOXIOUS HEAT THRESHOLD OF MICE MEASURED BY AN INCREASING-TEMPERATURE WATER BATH

Reproducibility of, and the effects of drugs on, the noxious heat threshold of the mouse tail. The three consecutive heat threshold measurements in the saline-treated (ip.) mice (two control measurements separated by a 20-min. interval plus one 30 min. after saline) gave the following values without a statistically significant difference: 45.80±0.13, 45.79±0.13 and 45.88±0.11°C (n = 106). These highly comparable values indicate both the remarkable reproducibility of the heat threshold upon repeated determinations and a lack of effect of saline injection on the heat threshold. Comparing the control heat thresholds in different groups of mice also resulted in minor differences. All three reference analgesics increased the heat threshold after intraperitoneal administration. Morphine (3–24 mg/kg) proved the most efficacious and most potent agent as it induced the highest elevation of the heat threshold approaching 5°C, and its minimum effective dose was the smallest (6 mg/kg). The two non-steroidal anti-inflammatory analgesics diclofenac (3–30 mg/kg) and metamizol (dipyrone, 100–1000 mg/kg) exerted smaller effects (0.69 and 1.23°C elevation of the threshold) only at the highest dose applied. The two higher doses of diazepam (15 and 30 mg/kg) also evoked an elevation of the heat threshold, while droperidol did so only at the highest dose (7.5 mg/kg). The maximum heat threshold elevation produced by these drugs was 1.38 and 1.21°C.

Effects of drugs on the psychomotor activity in the open field test. Morphine (3–24 mg/kg) decreased the number of rearings without affecting any other activity in the open field test. Metamizol (100–1000 mg/kg) exerted a marked and apparently dose-dependent inhibitory effect by inhibiting most measured parameters with the lowest dose having only minor influence restricted to the last third of the examination interval. Similarly, diazepam (5–30 mg/kg) showed apparently dose-dependent inhibitory actions. Not unexpectedly, droperidol (0.75–7.5 mg/kg) exerted a marked and apparently dose-dependent inhibitory effect on all parameters of the open field test. It should be stressed that none of the drugs or combinations induced general anaesthesia or even an inability of the animals to show a nocifensive response.

Effects of drug combinations on the heat threshold and in the open field test. The smallest applied dose of morphine (3 mg/kg) that failed to elevate the noxious heat threshold and that was without effect in the open field test was combined with diclofenac (10 mg/kg), metamizol (500 mg/kg), diazepam (5 mg/kg) or droperidol (2.5 mg/kg), at doses that were also subliminal regarding the antinociceptive action (heat threshold elevation). All these combinations induced a statistically significant elevation of the noxious heat threshold as compared to the pre-dose values. Combinations of morphine with the other analgesics tested evoked no or apparently less psychomotor inhibition than the analgesic alone while combinations of morphine with a psychoactive drug appeared to evoke slightly more inhibition than diazepam or droperidol alone. In contrast, combination of the two non-opioid analgesics (diclofenac and metamizol) failed to alter the heat threshold.

The increasing-temperature water bath seems suitable for reproducible measurement of the noxious heat threshold of the mouse tail and assessment of the thermal antinociceptive action of analgesics as well as for testing their interactions. As the heat threshold is a parameter that is routinely determined in electrophysiological experiments and assessment of experimental heat pain in humans, the present model can be considered more translational than the conventional thermonociceptive methods based on latency measurement. As most conventional thermonociceptive tests (hot plate, tail-flick, plantar test) are largely insensitive to various non-steroidal anti-inflammatory drugs (Le Bars et al., 2001; Bölcskei, 2012), the ability of the heat threshold measurement approach to detect the thermal antinociceptive effect of diclofenac appears an advantage. The method used is able to detect positive antinociceptive interactions between morphine on the one hand and non-opioid analgesics (diclofenac, metamizol) or psychoactive drugs (diazepam, droperidol) on the other hand. Furthermore, our results also emphasize the importance of the inclusion of behavioural studies, at least an open field test, in the preclinical testing of antinociceptive drug effects, which has previously been pointed out by various reviews (Negus et al., 2006; Mogil, 2009).

SUMMARY OF THE NEW FINDINGS

1. Balneotherapy with sulphurous medicinal water improved the psoriatic symptoms as shown by a reduced PASI from 7.74±1.60 to 3.81±1.02. The SST-like immunoreactivity of the plasma was significantly elevated at the end of the three week long therapy (1st day: 10.07±0.61 fmol/ml; 21st day: 21.26±2.09 fmol/ml). At the end of the therapy in the dermis reduced amount and in the epidermis normal distribution of Langerhans cells was observed.
2. In oxazolone-induced mouse model of allergic contact dermatitis swelling was reduced by Harkány medicinal water. The level of MPO and TNF-α did not change in the inflamed skin samples. Harkány medicinal water did not influence the histopathological changes. Therefore, it can be concluded that sulphurous medicinal water treatment does not influence the cellular phase of the inflammation remarkably, but it acts on the vascular phase. The 37 ^{o}C distilled or tap water did not influence the inflammation, therefore, we suggest that the effectiveness of medicinal water can be determined by chemical components, mainly by hydrogen-sulphide.
3. In MIA-induced mouse model of osteoarthritis significantly less decrease of weight distribution was observed in the medicinal water-treated group. However there was no significant difference in the change of mechanonociceptive threshold between the medicinal water-treated and control group, the weight distribution data suggest the pain is reduced by balneotherapy. According to the histopathological investigations inflammation and cartilage degeneration decreased in the medicinal water-treated group.
4. The two-week-long bath treatment with H_2S donor NaHS solution significantly increased the plasma level of SST. Supposedly, the endogenous anti-inflammatory and analgesic

neuropeptide SST plays a role in the anti-inflammatory and analgesic mechanism of sulphurous medicinal water.

5. In the animal model of OA reduced hyperalgesia less decrease of weight distribution was observed in the Trpa1 gene-deficient mice. Consequently, TRPA1 has a role in the transmission of nociceptive stimuli in mouse model of osteoarthritis.

6. Nonivamide (0.01%) was found as the only identified capsaicinoid molecule in the commercially available EMSPOMA® cream, which is in use in clinical practice.

7. We provided evidence that topical therapy with nonivamide-containing cream has analgesic effect in chronic low back pain. The 30 min. treatment increases SST-IR in the human plasma. However, in animal studies our workgroup proved that SST level of the plasma significantly increased by stimulation of capsaicin-sensitive nerve endings, present data first demonstrated that the local capsaicinoid (nonivamide) treatment exerts systemic analgesic effect, while the plasma level of anti-inflammatory and antinociceptive peptide SST shows threefold elevation (from 16.8±3.1 fmol/ml to 56.9±7.7 fmol/ml).

8. The increasing-temperature water bath was proved suitable for reproducible measurement of the noxious heat threshold of the mouse tail and assessment of the thermal antinociceptive action of morphine and diclofenac as well as for testing the morphine-sparing effect of diclofenac, metamizol, diazepam and droperidol. Some findings support the view that even a considerable psychomotor inhibition *per se* causes no thermal antinociceptive effect in the present model. The study also indicates that antinociceptive effects may not be assessed separately but should probably be accompanied by quantifying psychomotor activity.

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LIST OF PUBLICATIONS

Original full publications the thesis is based upon

Boros M, Kemény Á, Sebők B, Bagoly T, Perkecz A, Petőházi Z, Maász G, Schmidt J, Márk L, László T, Helyes Zs, Szolcsányi J, Pintér E. **Sulphurous medicinal waters increase somatostatin release; it is a possible mechanism of anti-inflammatory effect of balneotherapy in psoriasis.** European Journal of Integrative Medicine 2013;5:109-118.
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Other publications

Nagy L, Filotas D, **Boros M**, Pozsgai G, Pinter E, Nagy G. **Amperometric cell for subcutaneous detection of hydrogen sulfide in anesthetized experimental animals.** Physiological Measurement 2014;35**:**2475-2487.
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