THE TRPA1 MEDIATED EFFECTS OF HYDROGEN SULFIDE AND POLYSULFIDE COMPOUNDS ON ANIMAL MODELS OF ACUTE AND CHRONIC INLFAMMATION

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INTRODUCTION

1.1. H_2S, a novel gasotransmitter

In the early 1990s H_2S was recognized as the third gasotransmitter apart from NO and CO. Previously H_2S was only regarded as a toxic gas, but soon many of its pleiotropic biological roles emerged such as it's role in nociception, inflammation and regulating the vascular tone. Its potential biological effect on capsaicin-sensitive sensory nerves was first suggested in the lung [1]. Further insight was gained about their relationship through animal experiments regarding the urogenital system [2,3]. Although, there were already in vitro data available on the effects of H_2S utilizing TRPA1 expressing CHO cells, Miyamoto's workgroup was the first to demonstrate it's effects on DRG cells that express the receptors naturally[4]. The CGRP mediated vasodilative effect of H_2S was both decreased in TRPA1 KO mice and WT mice receiving HC030031[5–7].

1.2. The chemical and biological interactions between H_2S and NO

The research conducted by Murand and Ignarro lead to the remarkable recognition, that NO is produced endogenously and is capable of modulating signalling pathways albeit it's physical and chemical properteis[8,9]. Their ground-breaking discovery reverberated with the scientific community and suddenly the field of gasotransmitters started to expand rapidly. Kimura et al. was the first to describe the connection between the two gasotransmitters H_2S and NO and their synergistic dilative effect on isolated aortic segments[10]. Ever since these initial findings there has been extensive effort from the researchers to elucidate the complex interaction between the two molecules and their signalling pathways[11]. This task is further burdened by the fact that apart from their pharmacodynamic interactions the two molecules can also react with each other chemically[12]. The chemical reactions can produce an intricate network of intermediary products, which further complicates our understanding of the phenomenon, therefore it is not surprising that many conflicting results has surfaced regarding the topic since it's advent[12]. The rapid, intertwined and quite too often reversible nature of the chemical reactions has made it difficult to identify the exact products that are responsible for the observable biological effects. Some of these different bioactive intermediates include nitrosopersulfide (SSNO), H_2S_n and dinitrososulfite (SULFI/NO) nitroxyl (HNO), nitrous oxide (N_2O), nitrates, sulfites and sulfane sulfur[12].

1.3. The role of neuronal TRPA1 channels regarding inflammation and pain.

Expression of TRPA1 channels by somatic and visceral primary sensory neurones is well known. A subset of peptiderg nociceptors, also known as capsaicin-sensitive sensory neurones, co-express TRPV1 and TRPA1 channels[13]. Activation of these channels induces membrane depolarization by Na^+ influx, while the inward Ca^{2+} current increases the intracellular Ca^{2+} concentration, which is followed by the release of neuropeptides such as CGRP, substance P (SP) and neurokinin A[14]. These subsequently released pro-inflammatory neuropeptides amplify nociception and mediate neurogenic inflammation and are responsible for the local efferent function of the capsaicin-sensitive sensory nerve endings including vascular changes such as vasodilatation and plasma protein extravasation followed by recruitment of inflammatory and immune cells[15,16]. Following the activation of TRPA1 channels, the release of pro-inflammatory mediators is also accompanied by the release of anti-inflammatory peptides such as somatostatin which are responsible for the systemic efferent function of the capsaicin sensitive nerve ending[17]. Somatostatin is a cyclic peptide that is present in both the central nervous system and in the peripheral tissues. Neuronal derived somatostatin is capable of exerting systemic anti-nociceptive and anti-inflammatory effects acting mainly on the sst4 receptor[15]. In line with the previous observations DMTS potently attenuated the hyperalgesia and inflammation in mice due to mild heat injury acting through the TRPA1-sst-sst4 axis[17].

1.4. Effects of sulfur species mediated by TRPA1 channels

The effects of H_2S on the TRPA1 was most commonly verified by the application of specific receptor inhibitors (AP18, HC030031) or by the means of genetic ablation of the receptor in mice[4,5,18]. Furthermore the cysteine groups of the receptor responsible for it's activation by sulfide compounds were successfully identified[19].

The TRPA1 mediated effects of polysulfides, were observed on a cellular level utilizing RIN14B cells, the chemosensitive thoracic aorta cells of the species gallus domesticus and also on DRG neurons isolated from mice[18,20,21]. The polysulfide evoked Ca^{2+} signals were sensitive to TRPA1 antagonists and were diminished in the absence of the receptor. In vivo, the intraplantary injected sodium polysulfide induced oedema and hyperalgesia in a TRPA1 dependent manner in mice[21].

Despite the extensive research that was conducted regarding the role of the TRPV1 and TRPA1 receptors in inflammation and pain and their connection to the gasotransmitter H_2S

this vast amount of information has still left us with unanswered questions. Therefore it is of pivotal importance to further expand our knowledge of this intriguing topic.

AIMS

The discovery that H_2S is produced endogenously and functions as a gasotransmitter, has opened up the path to further research focusing on understanding it's exact biological nature. Early on it was clear that H_2S bears a complex role in the biological redox systems. Unfortunately, already at the beginning of the investigations, it was obvious that H_2S can lead to the formation of a diverse range of elusive intermediary mediators. At the present, even such a fundamental question as measuring the H_2S levels in biological sample is surrounded by intense debate. In our experiments we intended to investigate the effects of three different kind of hydrogen sulfide donor compounds, utilizing an acute and a chronic inflammation animal model.

In the case of K/BxN serum transfer arthritis model we aimed at finding answers to the following questions:

1. What is the influence of GYY4137 treatment on the severity of the developing arthritis?
2. Are there any observable differences between the TRPA1 and sst4 gene knock out mice and their respective wild type counterparts regarding the progression of the arthritis?
3. Is it possible for the GYY4137 to activate the TRPA1 receptor in vitro, if so, what could be the underlying mechanism?
4. Is it possible for the GYY4137 to induce the release of anti-inflammatory neuropeptides from the nerve ending found in the skin ex vivo?

In the carrageenan evoked acute inflammation model we focused on investigating the effects of the inorganic polysulfide and the organic polysulfide DMTS regarding the following aspects:

1. What is the effect of DMTS or POLY treatment on the mechanical pain threshold?
2. Is oedema formation influenced by the DMTS or POLY treatment?

3. Is there a difference in the myeloperoxidase activity or the extravasation measured by in vivo optical imaging due to the DMTS or POLY treatment?
4. Are the effects of DMTS or POLY treatment influenced by the lack or presence of the TRPA1- or sst4 receptors?

EXPERIMENTAL MODELS AND INVESTIGATIONAL TECHNIQUES

3.1. Animals

Experiments were conducted on genetically modified male mice lacking functional TRPA1 or sst4 receptors (KO) and their wild-type counterparts (WT; 2–4 months, 20–25 g) (27, 31). Age-matched animals were used in the study. The original heterozygous TRPA1 breeding pair was a generous gift from Pierangelo Geppetti (University of Florence, Italy) and the original sst4 mice was made available to us by Jeremy P. Allen and Prof. Dr. Pierce C. Empson[22,23]. These mice were originally generated and characterized by Bautista and colleagues (31). Neither the strain with genetic modification of TRPA1 nor that with modified sst4 gene is available commercially. TRPA1 and sst4 WT and KO breeding lines were produced by crossing respective heterozygote animals. WT and KO animals were chosen from the resulting litter and used for further breeding. Animals were bred and kept in the Laboratory Animal Centre of University of Pécs under standard pathogen free conditions at 24–25°C, 12 h light/dark cycles. Mice were housed in groups of 5–10 in polycarbonate cages (330 cm2 floor space, 12 cm height) on wood shavings bedding. Animals were provided standard diet and water ad libitum.

3.2. 2. Ethics statement

All experimental procedures were carried out according to the European Communities Council Directive of 2010/63/EU. The studies were approved by the Ethics Committee on Animal Research, University of Pécs (license numbers: BA02/2000-47/2017 and PE/EA/3895-6/2016).

3.3. Experimental models

3.3.1. Carrageenan-Induced Hind Paw Inflammation

Inflammation of one hind paw was triggered by intraplantar injection of carrageenan (20 µL, in saline 3%). The contralateral paw received saline [24]. The side of carrageenan injection was randomized. Animals were treated with either POLY (17 µmol/kg, i.p.) or DMTS (250 µmol/kg, i.p.) or the respective vehicle 30 min before challenge of the paws and every 60 min

afterward (seven times altogether). POLY was prepared freshly before each application. DMTS was prepared daily.

3.3.2. K/BxN Serum-Transfer Model of Autoimmune Arthritis

Kouskoff et al. generated the K/BxN spontaneously arthritic mouse strain with distinct features representing human rheumatoid arthritis [25]. Transferring sera from K/BxN mice into non-arthritic mouse strains results in a similar arthritic condition as described in the donor K/BxN strain. In a comprehensive review, Christensen et al. summarized the underlying pathogenesis and distinct features of the K/BxN serum-transfer arthritis model [26] Briefly, in our protocol, serum-transfer arthritis was induced by a single intraperitoneal injection of 300 µL of serum acquired from K/BxN mice. The non-arithrogenic control serum was obtained from BxN mice. Prior to the induction of arthritis, mice received a single treatment of slow-releasing sulfide donor GYY4137 or PBS. The treatment with GYY4137 (50 mg kg−1 day−1) or PBS was continued daily for 7 days after serum transfer. In the case of TRPA1 WT and KO mice we divided them into two additional groups. One set of mice were sacrificed on day three for subsequent cytokine measurements performed on samples obtained from their hind paws. The other set of mice were used for the functional, in vivo imaging and histological measurements. Regarding the sst4 mice, all measurements were performed the same way as it was described with the TRPA1 mice except for the cytokine measurements.

3.4. Investigational techniques

3.4.1. Preparation of inorganic polysulfide and DMTS solutions

Polysulfide was prepared as described earlier [27]. Stock solutions of hypochlorous acid and sodium sulfide nona hydrate were prepared in distilled water using polypropylene tubes blown with nitrogen gas beforehand. All later dilutions and reactions were performed in similar tubes. Reagents were kept on ice. Concentration of hypochlorous acid was calculated from the light extinction of the solution at 292 nm wavelength ($E292 = 350\ M^{-1}cm^{-1}$). Concentration of sulfide was derived from the extinction at 230 nm ($E230 = 7700\ M^{-1}cm^{-1}$) and the reaction with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB). Extinction of the reaction product of sulfide and DTNB was measured at 412 nm ($E412 = 28{,}200\ M^{-1}cm^{-1}$). Sulfide concentration was calculated as the mean of the two values yielded by direct spectrophotometry and reaction with DTNB. Stock solutions of hypochlorous acid and sulfide were prepared daily. Sulfide stock solution was diluted further in distilled water to 60 mM. Hypochlorous acid solution was added slowly under stirring to produce 20 mM in the final volume. The reaction of sulfide and hypochlorous acid produces POLY and the solution was made both isohydric, and isotonic by the addition of distilled water, PBS and hydrochloric

acid. DMTS does not dissolve in water readily, therefore it had to be diluted in multiple steps. Final DMTS solutions contained 2.24% v/v DMSO and 0.45% v/v polysorbate 80. Vehicle had 2.5% v/v DMSO.

3.4.2. Determination of polysulfide concentration via cold cyanolysis

Concentration of polysulfide present in our solutions regarding the POLY used in the carrageenan model and also the ones generated from GYY4137 was measured by cold cyanolysis, as described earlier [28].

3.4.3. Measurement of Ca^{2+} influx into CHO Cells expressing Human TRPA1 by flow cytometry.

Ca^{2+} influx measurements were performed on native or human TRPA1 expressing chinese hamster ovary cells, (CHO). For each measurement approximately 10^4 cells were resuspended in 100 µl and incubated with Fluo-4AM fluorescent dye (Invitrogen, 0.4 µL, 1ug μL^{-1} dissolved in DMSO) for 30 minutes at 37 ^{o}C. Mean green fluorescence of the samples was compared with base fluorescence of dye-loaded control cells, resulting in a baseline value of 1. Some cell groups were also pre-incubated with selective TRPA1 receptor antagonist HC-030031 (50 µmol L−1 in ECS) for 5 min. We measured the TRPA1 activating potential of hydrogen sulfide and inorganic polysulfide produced from either hydrogen sulfide or GYY4137. Control measurements were performed with the appropriate vehicle solutions, respectively.

3.4.4. Detection of Somatostatin Release From Murine Nerve Endings by Radioimmunoassay

TRPA1 WT and KO mice were sacrificed by cervical dislocation. Hind legs were shaved by a fine clipper. The skin was removed and placed in oxygenated synthetic interstitial fluid at room temperature. Skin samples were fixed inside-out on acrylic rods by sutures and were transferred to 37°C oxygenated SIF solution in a shaking bath and incubated for 30 minutes. The quantitative determination of the released somatostatin from the basal, stimulated and post stimulated samples were performed with radioimmunoassay (RIA)[17]. Each sampling phase was performed after 5 minutes of incubation period respectively, and the stimulations were performed with 10 µmol L^{-1} POLY or vehicle in the presence or absence of 50 µmol L^{-1} HC030031.

3.4.5. Detection of inflammatory cytokines in the K/BxN serum transfer model.

Briefly, after induction of arthritis, on day 3, mice were anesthetized with ketamine/xylazine (100/10 mg kg^{-1}) and were sacrificed via cervical dislocation. Subcutaneous tissue of the hind paws was flushed immediately with lavage fluid. Samples were then snap frozen in liquid

nitrogen and were stored at −80°C. Quantitative determination of IL-1β, KC, MIP-1α, and MIP-2 concentrations from the samples were performed using MILLIPLEX MAP Mouse Cytokine/Chemokine Magnetic Bead Panel—Immunology Multiplex Assay (Merck Millipore, Billerica, MA, USA). A Luminex 100 device was used for the measurement, and the interpretation of data was performed with the Luminex 100 IS software[29].

3.4.6. Measurement of mechanonociceptive thresholds

Mechanical hyperalgesia of the hind paws was determined by dynamic plantar aesthesiometry (DPA, Ugo Basile 37400, Comerio, Italy) [17].

3.4.7. Detection of hind paw volume

Volumes of the hind paws were measured by plethysmometry (Ugo Basile Plethysmometer 7140, Comerio, Italy) [24].

3.4.8. Drop latency/grip test

A simple grip test was performed as following. Mice were placed on a wire grid, which was then lifted up in horizontal position and rotated 180° around its horizontal axis, leaving the animal in an upside-down position and forcing it to hold onto the grid against its bodyweight. Healthy mice are able to cling to the grid for at least 20 s.

3.4.9. Arthritis score

The extent of oedema formation and hyperaemia of the hind limbs were evaluated on days 3, 5, and 7 according to a semi quantitative clinical score system (0–1.5, healthy condition; 1.5–2.5, minimal signs of disease; 2.5–4, mild inflammation; 4–7, moderate inflammation; and 7–10, severe inflammation) [30]

3.4.10. *In Vivo* Luminescence and Fluorescence Imaging

I.p. luminol sodium salt (150 mg kg^{-1}) was used to detect production of reactive oxygen species (ROS) correlated with neutrophil MPO activity. Furthermore, i.v. IR-676 (0.5 mg kg^{-1}) fluorescent dye was used to assess plasma protein extravasation. Bioluminescence was measured for 10 min and fluorescence for 20 min post injection using the IVIS Lumina II (PerkinElmer, Waltham, USA) [31].

3.4.11. Histological evaluation

Tibiotarsal joints were harvested on day 7. Samples were fixed in 4% buffered paraformaldehyde and then decalcified and embedded in paraffin. Sections of 3–5 μm were produced and stained with haematoxylin and eosin. Histopathological changes were scored (0–3) by a blinded independent expert. Factors taken into consideration were the followings:

cartilage destruction, mononuclear cell infiltration, synovial cell proliferation, fibroblast number, and collagen deposition. A composite arthritis score (ranging 0–12) was created [30,31].

3.4.12. Statistical analysis

Data are presented as mean ± standard error of the mean. Results were analysed by one-way or two-way analysis of variance (ANOVA) followed by Tukey's, Dunnett's, or Bonferroni's test. Data of SOM release were analysed by Kruskal–Wallis test due to deviation from normal distribution in various tests. Scatterplots of data on mechanical pain threshold, arthritis score, suspension time, and hind paw volume show all individual data points. Box plots on histological scores show median values (horizontal line), minimal and maximal values (whiskers), and the 25th and 75th percentiles (box). Histological score values were analysed by Kruskal–Wallis test followed by Dunn's test. Concentration–response curves were fitted with four-parameter equation Statistical analysis were performed by GraphPad Prism 5 software.

RESULTS

5.1. The effects of GYY4137 slow release hydrogen sulfide donor on the K/BxN serum-transfer model of autoimmune arthritis in TRPA1 and sst4 KO mice.

5.1.1. Addition of hypochlorous acid to GYY4137 leads to formation of polysulfide which evokes Ca^{2+} influx in TRPA1 expressing CHO cells.

Sulfide was released from acidified solution of GYY4137. The GYY4137 solution after 90-min incubation with citrate buffer (pH 3.0) followed by the addition of 100 μmol L^{-1} of hypochlorous acid resulted in the formation of 77.15 ± 9.85 μmol L^{-1} of POLY. POLY (10 μmol $L^{-1)}$ activated CHO cells expressing human TRPA1. TRPA1 activation by POLY was prevented by HC-030031 (50 μmol L^{-1}) and was absent in cells not expressing the ion channel. Neither GYY4137 solution acidified with hydrochloric acid nor hypochlorous acid produced calcium influx compared with allyl isothiocyanate. Hydrogen sulfide only elicited Ca^{2+} signals at extreme high concentrations in both native and TRPA1 expressing CHO cells.

5.1.2. Sodium polysulfide stimulates somatostatin release from nociceptor nerve endings in a TRPA1-dependent manner

Skin flaps of TRPA1 WT mice responded to stimulation with 10 μmol L^{-1} of POLY by releasing 0.1407 ± 0.044 fmol mg^{-1} of somatostatin-like immunoreactivity (SOM-LI). Treatment of the samples with 50 μmol L^{-1} of HC-030031 and genetic lack of TRPA1 ameliorated SOM-LI liberation.

5.1.3. GYY4137 aggravates mechanical hyperalgesia in arthritic TRPA1 KO mice and ameliorates it in TRPA1 WT mice.

On day 3, GYY4137 aggravated mechanical nociception in arthritic TRPA1 KO animals than did both vehicles of GYY4137 in TRPA1 KO animals and TRPA1 WT GYY4137-treated ones. On day 7, GYY4137 treatment ameliorated mechanical pain in TRPA1 WT animals compared with KO ones.

5.1.4. GYY4137 lowers arthritis score in TRPA1 WT animals.

The score of TRPA1 WT vehicle-treated mice undergoing arthritis was higher on day 5 than that of their GYY4137-treated counterparts.

5.1.5. Genetic lack of TRPA1 impairs hanging performance in mice injected with BxN serum irrespective of GYY4137 administration

TRPA1 KO mice injected with inactive BxN serum exhibited statistically shorter suspension on days 5 and 7 when treated with vehicle than the respective TRPA1 WT groups.

5.1.6. GYY4137 does not influence body weight and hind paw volume detected by plethysmometry

Arthritis led to weight loss and the formation of oedema in all animal groups. Neither differences of treatment nor those of genotype revealed any influence on the course of body weight changes or on the swelling of the paws.

5.1.7. Sst4 receptors do not mediate protective effects of GYY4137

No differences in mechanical pain threshold, hanging performance, arthritis score, or body weight were found between GYY4137-treated sst4 WT and KO mice undergoing K/BxN arthritis. Similarly, no influence of the genetic lack of sst4 receptor was detected in arthritic mice receiving vehicle of GYY4137.

5.1.8. GYY4137 treatment increases neutrophil granulocyte accumulation and plasma extravasation in arthritic TRPA1 KO mice

Daily treatment with GYY4137 elevated bioluminescence at day 2 in TRPA1 KO animals compared with TRPA1 WT GYY4137-treated mice and with TRPA1 KO vehicle-treated ones as well. Plasma extravasation was aggravated by GYY4137 injections in TRPA1 KO animals

in relation to TRPA1 WT GYY4137-treated mice and to TRPA1 KO vehicle-treated animals at both 2 days and 6 days.

5.1.9. Genetic lack of sst4 somatostatin receptor does not influence myeloperoxidase enzyme activity and plasma extravasation in arthritic mice.

No difference in MPO activity detected by luminescence imaging was recorded between arthritic sst4 receptor WT and KO GYY4137-treated animals. No participation of the receptor showed up when comparing sst4 WT and KO mice receiving vehicle of GYY4137. Similar data were generated when detecting plasma extravasation by fluorescence imaging.

5.1.10. GYY4137 elevates MIP-2 level in inflamed hind paws of TRPA1 KO mice

Serum-transfer arthritis did not affect concentrations of IL-1β, KC and MIP-1α in the subcutaneous flushing fluid of the hind paws

. In case of MIP-2, larger concentrations were measured in TRPA1 KO GYY4137-treated animals compared with WT GYY4137-treated ones

5.2. Effects of sodium polysulfide and dimethyl trisulfide on the carrageenan induced acute inflammation model in TRPA1 KO and sst4 KO mice

Results

5.2.1 Inhibition of carrageenan-evoked mechanical pain by POLY is TRPA1 and sst4 receptor-dependent

Carrageenan-injected paws of TRPA1 WT and KO mice undergoing vehicle administration developed significantly lowered mechanical pain threshold compared to saline-treated ones. POLY significantly reduced mechanical hyperalgesia in carrageenan-injected feet of TRPA1 WT and sst4 WT animals in comparison with those of vehicle-treated ones. Inhibitory effect of POLY on mechanical nociception in carrageenan-treated hind paws was lacking in KO animals compared to WT ones.

5.2.2. No exclusive role of TRPA1 ion channel in the protective effect of DMTS in carrageenan-induced mechanical hyperalgesia

Carrageenan-treated hind paws of TRPA1 WT mice undergoing DMTS administration showed significantly less hyperalgesia than those administered vehicles. Protective effect of DMTS was reduced in carrageenan-injected feet of TRPA1 KO animals compared to those of TRPA1 WT ones. However, DMTS still alleviated mechanical hyperalgesia in carrageenan-

treated feet of TRPA1 KO mice at 2 and 4 h after challenge in comparison with vehicle-treated animals. Carrageenan-treated hind paws of sst4 receptor WT mice injected with DMTS developed significantly smaller hyperalgesia than those of vehicle-treated control animals. This effect of DMTS was absent among the sst4 KO mice.

5.2.3. POLY does not affect paw swelling evoked by carrageenan

Both vehicle and POLY-treated sst4 or TRPA1 WT and KO mice exhibited significant paw swelling upon carrageenan stimulation of the hind paws. POLY had no statistically significant inhibitory effect on the swelling of the feet in either animal groups.

5.2.4. Protective effect of DMTS in carrageenan-evoked paw swelling is independent of TRPA1, but is mediated through sst4 receptors

DMTS ameliorated swelling at 6 h in carrageenan-injected feet of TRPA1 WT mice compared to those of vehicle-treated ones. DMTS significantly relieved swelling in carrageenan-treated paws of TRPA1 KO mice at 4 and 6 h after challenge in comparison with those of vehicle-treated ones. DMTS relieved oedema formation in carrageenan-treated paws of sst4 WT animals at 6 h in comparison with those of vehicle-treated ones. DMTS did not show any protective effect in sst4 KO mice.

5.2.5. Carrageenan-evoked MPO activity of accumulated neutrophil cells is unaffected by administration of POLY

All animals developed significantly elevated MPO activity in carrageenan-injected hind paws. POLY did not ameliorate MPO activity in any of the animal groups.

5.2.6. DMTS inhibits MPO activity of accumulated neutrophil granulocytes independently of sst4 receptors

MPO activity in carrageenan-injected hind paws of DMTS-treated mice tends to be smaller than in those of vehicle-treated ones and the decrease proved only to be significant among the sst4 WT and KO mice.

DISCUSSION AND CONCLUSIONS

The main novel finding regarding the K/BxN serum-transfer arthritis is that the presence or absence of functional TRPA1 channels determines pronociceptive, pro-inflammatory, or, conversely, analgesic and anti-inflammatory effects of sulfide. Mice undergoing K/BxN

serum-transfer arthritis and lacking functional TRPA1 receptors showed more severe mechanical hyperalgesia, more expressed plasma extravasation, and MPO activity in the inflamed limb, as well as increased MIP-2 concentration in the affected paws when administered GYY4137. Alternatively, the slow-releasing sulfide donor ameliorated hyperalgesia, arthritis score, and histological cartilage destruction in TRPA1 WT animals. Another interesting point is that the protective effect was not mediated directly by sulfide itself but by polysulfides being formed out of it.

The protective role of TRPA1-mediated GYY4137 effect is pronounced by DPA measurement. WT animals showed elevated mechanical pain threshold, but we detected aggravated reaction in KO mice. K/BxN serum proved to be arithrogenic in all examined genotypes. GYY4137 caused significant reduction of the arthritis on day 5 in TRPA1 WTs.

In the hands of other authors, GYY4137 had an anti-inflammatory action in CFA-induced murine arthritis [32]. GYY4137 inhibited mediator release from activated human synoviocytes and articular chondrocytes as well[33]. These previous data are in concordance with our present results: TRPA1 WT mice showed milder mechanical hyperalgesia, arthritis score, and cartilage damage in response to GYY4137.

Surprisingly, GYY4137 did not affect paw swelling measured by plethysmometry but increased plasma extravasation detected by fluorescence imaging of IR-676 in TRPA1 KO animals. Plethysmometry was executed on days 3, 5, and 7; fluorescence imaging was performed on days 2 and 6. The two methods detect different parameters. Plethysmometry provides information about the cumulative swelling—which means the equilibrium of oedema formation and resolution—since the initiation of inflammation. Fluorescence imaging characterizes the actual rate or velocity of plasma extravasation since the administration of the fluorescent dye (20 min). The other seemingly conflicting data are the elevated MPO activity and MIP-2 content of inflamed paws despite no change in histological mononuclear cell accumulation score. MPO activity and cytokine measurements were performed on days 2 and 3, while histological evaluation took place at a different stage of the inflammatory process on day 7. At this time, rather, mononuclear cells dominate, and neutrophil accumulation together with MPO activity ceases.

It might seem also contradictory how the activation of TRPA1 channels and an increase of [Ca2+]i in nociceptors could lead to an opposite effect than nociception and inflammation. One proposed mechanism is the release of inhibitory neuropeptides (e.g., somatostatin). SOM

release might be due to opening of TRPA1 or TRPV1 channels [17]. Elevated SOM plasma concentration in response to painful disease was detected in rodents and patients[17,34]. SOM possesses systemic antinociceptive and anti-inflammatory properties mediated by sst4 receptors. Sst4 receptors expressed in sensory neurons, lymphocytes, and vascular endothelial cells might contribute to the protective effect[15]. However, in the present model of arthritis, genetic lack of sst4 receptors did not diminish protective effect of GYY4137. It has to be noted that protective effect of GYY4137 on mechanical pain threshold was also not detected in sst4 WT mice. This might be explained by minor genetic differences between TRPA1 and sst4 genetically modified mouse strains together with compensatory changes of gene expression in genetically modified animals.

Several possible mechanisms could be supposed in the background of TRPA1-independent pro-inflammatory effect of GYY4137. The sulfide compounds are not specific for only for the TRPA1 receptor and may also influence other signalling pathways[35]. Various inflammatory animal models were reported to be augmented by sulfide or alleviated by disabled synthesis of the gasotransmitter. Our results indicate that TRPA1-mediated protective effects of H2S "overwrite" inflammatory and pro-algesic effects mediated via other pathways[36,37]. Detrimental effects only manifest in the absence of TRPA1.

According to our data, concentration of the chemoattractant MIP-2 (CXCL2) was elevated by GYY4137 in inflamed hind paws of arthritic TRPA1 KO animals on day 3. This finding is in alignment with increased MPO activity—denoting neutrophil granulocyte accumulation—in tibiotarsal joints of TRPA1 KO mice detected on day 2. MIP-2 is a chemoattractant for neutrophil cells and is released by monocytes, macrophages, epithelial cells, and hepatocytes[38].

From our K/BxN serum-transfer arthritis model related experiments we concluded that, the polysulfides generated from hydrogen sulfide are more potent activators of TRPA1 receptor by magnitudes. Furthermore previous studies had already proposed, that the effects of hydrogen sulfide are actually mediated by polysulfides following it's conversion[27,39]. Pozsgai et al, had already described the anti-nociceptive and anti-inflammatory effects of DMTS following mild heat injury, that was mediated by the TRPA1 and sst4 channel[17] Therefore we, decided to further investigate the role of DMTS and inorganic polysulfides in the pathomechanism of inflammation and nociception.

According to our data activation routes of the sensory neuron–somatostatin axis other than TRPA1 ion channels are in play in case of DMTS, as the organic trisulfide elicited antinociceptive effect and inhibited paw swelling independently of TRPA1, but still via sst4 receptors. Similar mechanisms might have been in play leading to the trend of inhibition of hind paw oedema detected by plethysmometry in TRPA1 KO mice treated with POLY. Several such alternative mechanism could exist. It has been documented that supraphysiological concentration of H_2S behaves rather as an activator, while normal concentration leads to inhibition of T-type Ca^{2+} channels found on nociceptors[40–42]. Voltage-gated K^+ channels are potential mediators of the effects of DMTS too because they are expressed on DRG neurons and it was reported that $K_{V4.3}$ channels are inhibited by H_2S through persulfidation[43,44]. It is possible that the DMTS, could act the same way on these channels and lead to the depolarization of DRGs with subsequent somatostatin release.

Sodium POLY is an anionic compound, thus it most probably cannot penetrate into the central nervous system. It reacts readily with cysteine amino acids of proteins and loses its negative charge[45]. However, proteins are excluded from the brain and cannot transport POLY there. This way the effects of POLY described in the present study might rely on a peripheral mechanism. Organic trisulfides such as DMTS are highly lipophilic and penetrate the blood–brain barrier freely[46]. An uptake via facilitated diffusion or active transport has been proposed in case of DMTS, too. Target proteins in the spinal cord and brain available for DMTS might contribute to its differing effect on nociception from that of POLY.

An inhibitory effect of DMTS on MPO activity was found that is mediated by neither TRPA1 nor SOM. Sulfide potentially being released from DMTS directly inhibits MPO activity of neutrophil granulocytes offering a straightforward mechanism[47]. There is also wide range of publications describing the inhibitory effect of H_2S regarding the neutrophils[48,49]. We conclude that activation of peptiderg sensory neurons, release of SOM and subsequent activation of sst4 receptors are important mediators of anti-hyperalgesic effect of both POLY and DMTS. Unlike POLY, DMTS possesses anti-inflammatory activity, too. The aforementioned mechanism contributes to the amelioration of oedema formation by DMTS complemented by other means of peptidergic-nerve activation as the effect depends on the presence of functional sst4 receptors. DMTS is able to suppress MPO activity of neutrophil granulocytes at the site of inflammation without the involvement of the sensory neuron–SOM axis.

Superior chemical stability, favourable pharmacokinetic properties, and significant translational potential—due to being a recognized food additive and having been patented as cyanide antidote—set DMTS in front of sodium POLY as a candidate of drug development.

SUMMARY OF THE NEW FINDINGS PRESENTED IN THE THESIS

In the first part of my thesis we proved that the GYY4137 derived hydrogen sulfide can be successfully converted into polysulfides *in vitro*. The produced polysulfides activated the TRPA1 receptors expressed by CHO cells. Furthermore polysulfide stimulation of skin preparations can lead to the release of somatostatin via TRPA1 receptors *ex vivo*. The beneficial, or detrimental effect of GYY41317 was determined by the presence or absence of the TRPA1 receptors, *in vivo*. In TRPA1 WT mice GYY4137 treatment proved to be anti-nociceptive and anti-inflammatory, in contrast the TRPA1 KO mice developed a more severe form of arthritis due to the sulfide donor treatment. Our experiments provide insight into the crucial role of the TRPA1 receptor in mediating the effects of hydrogen sulfide and inorganic polysulfides, unfortunately we were unable to find the same relevance for the sst4 receptor in the K/BxN serum-transfer arthritis model **(Table 1)**.

	GYY4137			
	TRPA1 WT	TRPA1 KO	sst4 WT	sst4 KO
Mechanical hyperalgesia	↓	↑	–	–
Paw swelling	–	–	–	–
Grip test	–	–	–	–
Arthritis score	↓	–	–	–
Neutrofil MPO activity	–	↑	–	–
Plasma extravazation	–	↑	–	–
MIP2α	–	↑	n.m.	n.m.
Histological score	↓	–	↑	–

Table 1: Influence of the TRPA1 and sst4 genes on the disease severity of GYY4137 treated arthritic mice. Legends: ↓: significantly decreased compared to vehicle treatment; **—:** same as the vehicle treated group; ↑: significantly increased compared to vehicle treated group; n.m.: not measured parameter.

In the second part of the thesis we have concluded the following novel findings utilizing the carrageenan induced acute inflammation model:

Anti-nociceptive effect of POLY in carrageenan-evoked paw inflammation depends on TRPA1 ion channel opening by the drug, release of SOM from the activated peptidergic sensory nerve fibres and subsequent activation of sst4 receptors, as the anti-hyperalgesic effect of POLY was absent in TRPA1 and sst4 KO mice.

Organic trisulfide DMTS possessed an anti-nociceptive effect not only in TRPA1 and sst4 receptor WT animals, but also in TRPA1 KO ones. Protective activity was significantly weaker in TRPA1 KO mice than in WT ones. These findings imply target molecules of DMTS on sensory nerve endings other than TRPA1 leading to activation of the fibres and SOM release.

Polysulfide administration exhibited no statistically significant effect on carrageenan-induced paw oedema, unlike DMTS that ameliorated swelling in TRPA1 WT, KO, and sst4 receptor WT mice. These data point toward other mediators of DMTS effect than TRPA1 on peptidergic nociceptors.

Polysulfide had no effect on MPO activity of the inflamed hind paws. Interestingly, DMTS significantly lowered MPO activity characterizing neutrophil accumulation in sst4 receptor WT and KO animals. A similar trend emerged in TRPA1 WT and KO animals not bestriding the limit of statistical significance. Our results indicate a mechanism of action of DMTS regarding MPO activity differing completely from the one suggested by data on mechanical nociception and paw swelling. Activation of TRPA1, release of SOM and its effect on sst4 receptors do not contribute to the inhibition of MPO activity by DMTS. **(Table 2)**.

	TRPA1 WT		TRPA1 KO		sst4 WT		sst4 KO	
	DMTS	POLY	DMTS	POLY	DMTS	POLY	DMTS	POLY
Mechanical hyperalgesia	↓	↓	↓	—	↓	↓	—	—
Paw swelling	↓	—	↓	—	↓	—	—	—

Neutrofil MPO activity	(↓)	–	(↓)	–	↓	–	↓	–

Table 2: Influence of the TRPA1 and sst4 genes on the effects of DMTS and POLY treatment on the acute inflammation evoked by carrageenan. of GYY4137 treated arthritic mice. Legends: ↓: significantly decreased compared to vehicle treatment; ─: same as the vehicle treated group; ↑: significantly increased compared to vehicle treated group.

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PUBLICATIONS

1. Publications related to the thesis:

Bátai, I. Z., Horváth, Á., Pintér, E., Helyes, Z., & Pozsgai, G. (2018). Role of Transient Receptor Potential Ankyrin 1 Ion Channel and Somatostatin sst4 Receptor in the Antinociceptive and Anti-inflammatory Effects of Sodium Polysulfide and Dimethyl Trisulfide. Frontiers in Endocrinology, 9. https://doi.org/10.3389/fendo.2018.00055 (IF:3.634)

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Ittzes, B., Weiling, Z., **Batai, I. Z.**, Kerenyi, M., & Batai, I. (2016). Atropine and

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Bátai I. Hemolysed blood constrict cerebral arteries. II. Pécs-Oklahoma Symposium (II. POS), Pécs, Magyarország, 2013.

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4. Poster presentations:

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