FINAL REPORT

on the grant OTKA PD 112171 ***"Investigation of the role of polysulfides, TRPA1 receptors and somatostatin sst4 receptors in animal models of inflammatory and neuropathic pain, as well as rheumatoid arthritis"***

Main findings

Our research financed by the grant investigated effects of polysulfide compounds on nociception and inflammation. Our working hypothesis, based on preliminary data and literature, was that polysulfides activate TRPA1 ion channels of sensory neurons. Activation of TRPA1 on nociceptor nerve endings and T lymphocytes might mediate antinociceptive and anti-inflammatory effects. One major factor behind these effects might be somatostatin (SOM) released from peptidergic sensory nerve endings. According to previus data, sst4 receptors on sensory neurons, lymphocytes and endothelial cells might transmit these effects of somatostatin. The two forms of polysulfide compounds tested were sodium polysulfide (POLY) and garlic-derived dimethyl trisulfide (DMTS). POLY can conveniently be synthetized by reacting sodium sulfide nonahydrate with hypochlorous acid. Concentration of the solution can be checked by cold cyanolysis. Organic DMTS was chosen because it is commercially available in chemically pure form and is stable. Studying DMTS is made relevant by the fact that scientific effort is invested in the USA to use the chemical for the treatment of mass cyanide intoxication related to terrorism. DMTS has been patented for intramuscular application for this indication, but has not been licenced yet for human treatment.

Activation of TRPA1 ion channels was examined in CHO cells expressing human TRPA1 receptors and in trigeminal ganglion (TRG) neurons of mice genetically lacking the channel. CHO cells were loaded with fluo-4-AM calcium-sensitive dye. Fluorescence was detected by flow cytometry. Inorganic polysulfide dose-dependently increased intracellular calcium concentration of TRPA1-expressing CHO cells. EC_{50} value was 5.29 µmol/L. Calcium signals induced by submaximal concentrations of POLY were inhibited by TRPA1 antagonist HC030031. No calcium elevation could be induced in CHO cells not expressing TRPA1 ion channels. DMTS produced similar results with an EC_{50} value of 37.72 µmol/L. The effect of DMTS was tested in two other cellular systems. CHO cells described above were subjected to automated patch-clamp. EC_{50} value was 18.46 µmol/L in this setting and responses could be abolished by HC030031. Activity of DMTS was investigated in trigeminal ganglion (TRG) neurons isolated from mice genetically lacking TRPA1 ion channels (KO) and wild-type controls (WT). TRG cells were loaded with fura-2-AM calcium-sensitive dye and elevations of intracellular calcium concentration were detected by ratiometric calcium imaging. DMTS produced intracellular calcium signals in a similar portion of TRG cells as TRPA1 agonist allyl isothiocyanate. Responses evoked by both allyl isothiocyanate and DMTS could be diminished by HC030031. No calcium signal could be induced in cells lacking TRPA1 ion channels. The above data indicate that both POLY and DMTS are able to activate TRPA1 ion channels.

The ability of TRPA1 activation on peptidergic nociceptor nerve endings to liberate SOM was examined in skin samples isolated from hind legs of TRPA1 WT and KO mice. Skin flaps were placed into synthetic interstitial fluid for 5 min. SOM content of the supernatant was measured by radioimmunoassay. Supernatant was replaced for another 5 min. The second solution contained POLY (10 µmol/L) or DMTS (100 µmol/L). In some cases the second fraction of interstitial fluid contained HC030031 (50 µmol/L). Supernatant was removed again after 5 min and fresh synthetic interstitial fluid was applied for the same interval. Radioimmunoassay is based on competition of SOM in the sample with marked SOM of known

amount for a specific antibody. SOM is marked by gamma-radiating iodine. Immune complexes and unbound peptides are separated and gamma radiation of both parts is counted. Using standard samples, SOM concentration of unknown samples can be calculated. The first supernatant represents baseline SOM liberation. This value was subtracted from those of the second and third supernatants. The sum of the latter two values indicates total SOM release. Both POLY and DMTS induced SOM release form skin samples of TRPA1 WT animals. SOM outflow was inhibited by HC030031. SOM release was reduced by genetic lack of TRPA1, too.

Measurement of sulfide in biological samples is complicated and normal physiological levels of the mediator are widely debated. We contributed to the development of a potentiometric device capable of detection of gaseous hydrogen sulfide in the headspace of biological samples. Lower detection limit of the device is submicromolar, enabling it to record implicated physiological concentrations. The device utilizes silver/silver chloride electrode. The surface of the cell is covered with a thin layer of basic buffer containing dicyanoargentate. Gaseous hydrogen sulfide dissolves in this layer and is trapped as silver sulfide changing the potential of the silver electrode. The apparatus successfully measured sulfide from the closed headspace of sodium sulfide nonahydrate solutions. Samples have to be acidified with sulphuric acid for sulfide to enter gaseous form. The detection was stable up to 35 °C and even above this temperature only slight deviation was noted. One hind paw of NMRI mice was injected with carrageenan (3%, 20 µL). Animals were sacrificed after 6 hours and paws were harvested. Minced paws were added to sulphuric acid inside the primed cell and sulfide release was detected. Smaller sulfide values were detected in inflamed paws than in intact controls. The apparatus proved to be useful in the detection of small concentration of sulfide. The shortcoming of the method is the destruction of the sample by the addition of sulphuric acid.

Antinociceptive and anti-inflammatory effects of POLY and DMTS were investigated in animal models of acute pain and inflammation. One hind paw of TRPA1 and sst4 WT and KO mice was injected with carrageenan (3%, 20 µL). Animals were treated with POLY (17 µmol/kg) or DMTS (250 µmol/kg) 7 times for 6 hours. Both POLY and DMTS reduced mechanical sensitivity of inflamed hind paws detected by dynamic plantar aesthesiometry at 2, 4 and 6 hours. Protective effect of POLY did not occur in mice lacking either TRPA1 or sst4 indicating pivotal involvement of these two proteins. Antinociceptive effect of DMTS was absent in sst4 KO animals, but it could still be found in TRPA1 KO mice. These findings propose that TRPA1-independet activation of peptidergic neurons by DMTS and subsequent SOM release are the mediators. Unlike POLY, DMTS relieved swelling of carrageenan-injected paws. Oedema formation was measured by plethysmometry at 2, 4 and 6 hours. Mechanism was identical to that of the antinociceptive action: sst4 KO mice did not respond, but TRPA1 KO ones did. Moreover, DMTS treatment inhibited myeloperoxidase (MPO) enzyme activity recorded by luminescent imaging using luminol at 6 hours. MPO activity indicates neutrophil cell accumulation. This effect appeared in sst4 WT and KO mice, suggesting a completely different mechanism without any participation of TRPA1 activation or SOM release from sensory nerve endings. Seltzer-type unilateral sciatic nerve lesion was performed in TRPA1 and sst4 WT and KO mice. Traumatic mononeuropathy was confirmed 7 days after surgery by detecting mechanical hyperalgesia of the hind paws by dynamic plantar aesthesiometry. In neuropathic animals the same treatment schedule was followed as in the carrageenan model above: mice received 7 injections of DMTS (250 µmol/kg) 60 min apart. DMTS exhibited antinociceptive effect in TRPA1 and sst4 WT mice, but this action did not occur in their KO counterparts. Antinociceptive effect of DMTS in the Seltzer mononeuropathy model relies on SOM release from sensoy nerve endings upon TRPA1 activation.

Interestingly, administration of 7 doses of POLY (50 µmol/kg) did not affect mechanical pain threshold in neuropathic mice. Acute DMTS treatment was examined in another animal model. One hind paw of TRPA1 and sst4 WT and KO mice was subjected to heat injury by submerging the foot of the anaesthetized animal into 51 °C water for 15 seconds. A single dose of DMTS (250 µmol/kg) was applied 30 min before heat challenge. Mechanical pain threshold of the hind paws was tested by dynamic plantar aesthesiometry in the following 60 min. In concert with the above findings, DMTS exerted antinociceptive effect in TRPA1 and sst4 WT mice, but not in KO ones. According to our data, both POLY and DMTS have potential for acute antinociceptive application, except in case of neuropathy where POLY remained completely ineffective. While the activity of POLY seems to rely on selective TRPA1 activation, that of DMTS is more promiscuous. Based on the less selective mechanism, DMTS is able to ameliorate inflammatory oedema and neutrophil accumulation beside pain.

The effect of POLY and DMTS was also investigated in more chronic settings. The primary animal model chosen was complete Freund's adjuvant (CFA)-induced polyarthritis based on reliability, simplicity and clinical relevance. TRPA1 and sst4 WT and KO animals were injected with CFA (1 mg/mL) in one hind paw and at the base of the tail. The injection to the tail was repeated the next day. Animals were treated with POLY (50 µmol/kg) or DMTS (250 µmol/kg) daily for 21 days. Development of methemoglobinaemia and consequent hypoxia limit the dose of polysulfides in case of sustained administration. This problem has recently been published regarding DMTS. Mechanical pain threshold, grip performance and paw swelling were measured by dynamic plantar aesthesiometry and plethysmometry on days 3, 6, 12, 15 and 21. Grip performance was expressed as time to failure in upside down hanging of the mice on a metal mesh. Acute rate of plasma extravasation and neutrophil accumulation were detected by fluorescent and luminescent imaging on days 2, 6, 9 and 14. Micellar V680 fluorescent stain was used to characterize plasma extravasation. Size of the micelles only allows tissue accumulation of the stain if permeability of blood vessels is increased. Unfortunately, neither POLY nor DMTS exhibited any effect on pain or inflammatory parameters in CFA-induced arthritis. We decided to use the slow-release sulfide donor GYY4137 in a shorter model of rheumatoid arthritis. The extremely reliable K/BxN serum-transfer arthritis model was utilized. TRPA1 and sst4 WT and KO animals were injected with K/BxN serum intraperitoneally. Mechanical pain threshold, paw swelling, grip performance and clinical appearance of the hind paws were recorded on days 3, 5 and 7 as described above. Rate of plasma extravasation and MPO activity were detected by fluorescent and luminescent imaging on days 2 and 6. Tibiotarsal joints were harvested on day 7 and histologically processed. Subcutaneous lavage fluid of the hind paws was collected on day 3 and cytokine levels were evaluated by ELISA. Surprisingly, arthritic TRPA1 WT and KO mice exhibited opposite changes in response to GYY4137 administration. Mechanical pain threshold, arthritis score and histological cartilage destruction in tibiotarsal joints were reduced in animals possessing functional TRPA1 ion channels. Conversely, TRPA1 KO mice showed elevated mechanical hyperalgesia, plasma extravasation rate, neutrophil accumulation and MIP-2 chemokine concentration in the lavage fluid. In previus experiments TRPA1 activation and SOM release from sensory nerve endings of isolated mouse skin could not be evoked by sodium sulfide nonahydrate solution. On the other hand, both of these processes are readily produced by sodium polysulfide. We tested if GYY4137 might function as polysulfide donor. Sulfide released from acidified GYY4137 was converted into polysulfide by the addition of hypochlorous acid as detected by cold cyanolysis. Acidic pH and neutrophil garnulocyte-derived hypochlorous acid are characteristics of inflamed tissues. GYY4137 might function as an inflammation-selective polysulfide donor. In the K/BxN serum-transfer arthritis model sst4 can be ruled out as mediator of protective effects because no changes were seen in sst4 WT and KO mice. Sst2 receptors might mediate these actions of

GYY4137. Sst2 receptors are readily expressed in macrophages. Macrophages are pivotal in K/BxN serum-transfer arthritis. Mice lacking these cells are resistant to the model. According to literature, non-canonical p38/Akt, CREB activation, PI3K/Akt/Sp1 signalling pathway, COX2 and NF-κB activation might be responsible for detrimental effects of GYY4137 in TRPA1 KO mice. In TRPA1 WT animals protective effects dominate over damaging ones. Our findings indicate that POLY and DMTS are rather suitable for acute treatment of pain. DMTS shows potency as anti-inflammatory agent, as well.

Deviation from the work plan

Due to difficult determination of the exact chemical composition of potassium polysulfide, sodium polysulfide was used throughout the study. Unlike stated in the work plan, SOM release in response to polysulfide stimulation was detected in murine tissue (skin of the hind paw) rather than rat trachea. Our radioimmunoassay SOM detection method was originally fitted for rat samples, but we developed the process further to be able to use murine samples. This complements our other findings that are all produced in mice. The idea of detection of sulfide/polysulfide from murine samples was dropped because it would not have contributed significantly to the knowledge on the mechanism and effects of polysulfides. However, we contributed actively to the development of a highly sensitive potentiometric detection method of sulfide. Due to ineffectiveness of POLY and DMTS treatments in CFA-induced arthritis, we declined from performing c-Fos immunohistochemistry in brain and spinal cord samples generated in this model.

Publications

Talks

"Analgesic Effect of Polysulfide Compound Dimethyl Trisulfide Is Mediated via TRPA1 Receptors" Pharmacology 2014, British Pharmacological Society, 12-19 December 2014, London UK

"Dialkil-poliszulfidok hatásai a nocicepció és gyulladás egérmodelljeiben" Joint Conference of the Hungarian Pharmacology, Anatomy, Microcirculation and Physiological Societies (FAME 2016), 1-4 June 2016, Pécs, Hungary

"A szomatosztatin szerepe a szerves triszulfid és poliszulfid vegyületek analgetikus és gyulladásgátló hatásában" Joint Conference of the Hungarian Physiological Society, Hungarian Society of Experimental and Clinical Pharmacology and the Hungarian Society of Microcirculation and Vascular Biology, 13-16 June 2017, Debrecen, Hungary

Posters

Federation of European Physiological Societies, 27-30 August 2014, Budapest, Hungary

Hungarian Pain Society IASP Chapter, 2-3 October 2014, Pécs Hungary

Neuropeptides 2015, European Neuropeptide Club, 26-30 September 2015, Aberdeen, UK

3rd European Conference on the Biology of Hydrogen Sulfide, 3-6 May 2015, Athens, Greece

79th Conference of the Hungarian Physiological Society, 27-30 May 2015, Szeged, Hungary

15th Biannual Meeting of the Hungarian Neuroscience Society, 22-23 January 2015, Budapest, Hungary

4th International Conference on the Biology of Hydrogen Sulfide, 3-5 June 2016, Naples, Italy

FENS Regional Meeting, 20-23 September 2017, Pécs, Hungary

13th World Congress on Inflammation, 8-12 July 2017, London, UK

Congress on Drug Innovation, 9-11 April 2018, Velence, Hungary

82nd Conference of the Hungarian Physiological Society, 27-30 June 2018, Szeged, Hungary

5th World Congress on Hydrogen Sulfide in Biology and Medicine, 31 May-3 June 2018, Toronto Canada

Papers

Bátai IZ, Horváth Á, Pintér E, Helyes Z, Pozsgai G. 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. Front Endocrinol (Lausanne). 2018;9:55. doi: 10.3389/fendo.2018.00055.

Pozsgai G, Payrits M, Sághy É, Sebestyén-Bátai R, Steen E, Szőke É, Sándor Z, Solymár M, Garami A, Orvos P, Tálosi L, Helyes Z, Pintér E. Analgesic effect of dimethyl trisulfide in mice is mediated by TRPA1 and sst4 receptors. Nitric Oxide. 2017;65:10-21. doi: 10.1016/j.niox.2017.01.012.

Filotás D, Bátai IZ, Pozsgai G, Nagy L, Pintér E, NagyG. Highly sensitive potentiometric measuring method for measurement of free H2S in physiologic samples. Sens Actuators B Chem. 2017; 243:326-331. doi.org/10.1016/j.snb.2016.11.102.

Manuscripts in progress

A review article on our research topic titled “Effects of sulfide and polysulfides transmitted by direct or signal transduction-mediated activation of TRPA1 receptors” has recently been accepted by the British Journal of Pharmacology. The paper is not available online yet.

A manuscript on the effect of slow-release sulfide donor GYY4137 in murine serum-transfer arthritis titled “TRPA1 ion channel determines beneficial and detrimental effects of GYY4137 in murine serum-transfer arthritis” has been prepared for publication.