

THE ROLE OF THE TACHYKININ SYSTEM AND THE ACTIVATION OF SENSORY NERVE ENDINGS IN BOWEL AND AIRWAY INFLAMMATION MODELS

PhD Thesis



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1. Introduction

1.1 Capsaicin sensitive nerve endings, their functions and the neurogenic inflammation

The capsaicin sensitive nerve endings have three roles: afferent, local and systemic efferent functions. The classic afferent function means that the sensory nerve ending sends signals into central nervous system evoked by a stimulus (capsaicin, heat, mechanical stimulus, pH change, etc.) and nociception arises; the pain is the subjective perception of it. Beside this, different neurotransmitters are also released from the activated nerve ending, which exert local and systemic effects. Vasodilation, plasma protein extravasation, inflammatory cell activation, edema (neurogenic inflammation) are caused. Such neurotransmitters are the substance P (SP), neurokinin A (NKA), neurokinin B (NKB) which belong to the tachykinin family. This can be considered the local efferent function of the capsaicin sensitive nerve endings. Somatostatin is also released from the activated sensory nerve endings into the circulation, which has an antiinflammatory and analgesic effect (Pintér & Szolcsányi 1996; Szolcsányi, Helyes, et al. 1998; Szolcsányi, Pintér, et al. 1998). This is the systemic efferent function of sensory nerve endings.

1.2 Transient Receptor Potential Vanilloid 1 (TRPV1) receptor

The Transient Receptor Potential Vanilloid 1 receptor is a nonselective cation channel activated by painful thermal stimulus (above 43 °C), acidic pH (Tominaga et al. 1998), numerous exogenic compound (capsaicin, resiniferatoxin, piperin, zingeron, gingerol, ethanol) (Szolcsányi 1983; Szallasi & Blumberg 1989; Patacchini et al. 1990; Szallasi & Blumberg 1990; Caterina et al. 1997; Liu et al. 2000; Dedov et al. 2002) as well as endogenous substances (anandamide, lipoxygenase products, N-oleoil-dopamine) (Hwang et al. 2000; Trevisani et al. 2002; Chu et al. 2003). TRPV1 receptor has been being successfully cloned about one and half decades ago (Caterina et al. 1997; Caterina & Julius 2001; Clapham et al. 2003). The channel is built up from six transmembrane domains; the fifth and sixth domain is connected by a short hydrophobic flexible chain which takes part in the pore formation. The receptor is active in tetrameric form (Clapham et al. 2003).

Upon its activation, many different signal transduction pathways are activated or inactivated, the most important outcome is the cell membrane depolarization caused by calcium influx. The calcium accumulation can cause the elevation of cGMP level in a specific manner (Wood

et al. 1988). The phosphorylation sites of PKC (Bhave et al. 2003; Dai et al. 2004; Premkumar et al. 2004), PKA (De Petrocellis et al. 2001; Rathee et al. 2002; Bhave et al. 2003), and Ca²⁺/calmodulin dependent protein kinase II (CaMKII) enzymes on the TRPV1 receptors (Wood et al. 1988) are found.

TRPV1 receptors have an important role in the transmission of painful stimuli (nociception) and their presence is also discovered in the central nervous system where they have a role in the integration of painful stimuli (Huang et al. 2002; Cui et al. 2006). The activation of TRPV1 receptors causes the release of tachykinins from the sensory nerve endings and they are functioning as one of the important regulator of tachykinin system, too.

1.3 Tachykinin system

The first member of the tachykinin family was discovered by von Euler and Gaddum. In the '80, the mammalian tachykinin family was expanded with new members such as substance K (neurokinin A, NKA according to the new nomenclature) and neuromedin K (now it is called neurokinin B, NKB), neuropeptide K and neuropeptide γ which are the variants of NKA (Kimura S. 1983; Nawa et al. 1984; Tatemoto et al. 1985; Kage et al. 1988).

The receptors were discovered and characterized along with the mediators (Masu et al. 1987; Yokota et al. 1989; Bielow et al. 1990). Tachykinins are released mostly from the capsaicin sensitive nerve endings but they can be detected in other tissues beside neuronal structures. Tachykinin receptors are G-protein coupled receptors. Three receptors are known until now (neurokinin 1, 2, 3), their affinity and specificity are different to the given tachykinins.

The tachykinins are involved in several physiological processes. The tachykinins released in the spinal dorsal root area are involved in nociception. They also influence the cholinergic and adrenergic signal transduction (Grant 2002), but their most prominent effect is the mediation of neurogenic inflammation. Neurogenic inflammation is characterized by local vasodilation, plasma extravasation, edema formation and accumulation of inflammatory cells. The tachykinins can evoke smooth muscle cell contraction by the direct or indirect degranulation of mast cells causing histamine release (Jancsó et al. 1967; Jancsó et al. 1968; Szolcsányi 1984; Szolcsányi 1996a; Barthó et al. 2004).

Substance P binds to NK1 receptor, the NKA to NK2 receptor while NKB to NK3 receptor preferentially; they act as full agonist on the preferred receptor. SP causes the increase of the permeability of the vessels, plasma protein extravasation, enhances the production of inflammatory cytokines and the chemotaxis of T cells. With this effect they facilitate the

accumulation of neutrophil granulocytes, degranulation of mast cells and proliferation of lymphocytes (Grant 2002). SP increases the secretion of salivary glands, gastrointestinal tract and pancreas (Lembeck & Starke 1968; Konturek et al. 1981). Furthermore, they foster the proliferation and wandering of endothelial cells and the formation of new vessels (Maggi 1995). NKA shows the biggest affinity toward NK2 receptors; causes plasma extravasation, smooth muscle contraction and stimulates inflammatory cells (De Swert & Joos 2006) like SP. NK2 receptors act mainly on the periphery but they can be found in central nervous system, too. The NK3 receptor which can be found mainly in central nervous system, binds NKB, can be detected on the periphery as well (Frossard & Advenier 1991), but their role in neurogenic inflammatory processes seems less prominent.

After 2000 the tachykinin family was expanded with the freshly discovered hemokinins encoded by the Tac4 gene (Zhang et al. 2000). Several products of this gene are synthesized in human with alternative splicing which are called endokinins (EKA, B, C, D) and hemokinins (HK). There is only one variant in mice and rats called hemokinin-1 (HK-1). These new members with the suggested receptor conformers and tissue-specific post-translational modifications build up a fine-tuned tachykinin system where many cell lines and signal transduction pathways are involved (Page 2004; Page 2005).

1.4 The role of neurogenic inflammation in the airways.

Airway inflammation can be studied in different animal models. The most used are the endotoxin (LPS) evoked airway inflammation (Helyes & Hajna 2012), or the cigarette smoke induced chronic obstructive pulmonary disease (COPD) model (Shapiro 2000). The LPS-induced inflammation is a neutrophil granulocyte dominated acute inflammation while the smoke exposure leads to chronic changes, causes emphysema and fibrosis (Churg et al. 2008). Inflammatory and immune cells are also capable to produce tachykinins upon certain inflammatory stimuli. The cellular tachykinins have important role in airway inflammation such as asthma and COPD (Groneberg et al. 2006). Pulmonary epithelial cells can express SP in viral infection (Stewart et al. 2008).

The preferred ligands for NK1 and NK2 receptors are SP and NKA respectively (Frossard & Advenier 1991; Regoli et al. 1994). Our research group confirmed previously that in acute lung inflammation the simultaneous activation of NK1 and NK2 receptors play role in the neutrophil accumulation and the NK2 receptors are responsible for the increased airway resistance (Elekes et al. 2007).

Since the discovery of the third preprotachykinin gene (Tac4), the number of tachykinins is doubled, numerous peptides had been identified in the peripheral organs in many species and broad spectrum effects had been described (Page 2004; Page 2005; Page 2006). Hemokinins and endokinins show significant selectivity and potency on NK1 receptors and may take part in inflammatory cell functions (Groneberg et al. 2006; Page 2006). SP-like endokinins which are expressed mainly peripherally are thought to be NK1 receptor agonists (Page 2004), but the tachykinin system seems to be much more complex than it was thought at the beginning. Several tachykinin-mediated processes show tissue specificity which make possible the unique interactive combination of the tachykinins and receptor configurations (Page 2006).

1.5 A neurogén gyulladás jelentősége a vastagbélben

Neurogenic inflammation and neuroimmune interactions have proved to take part in the development of IBD. It is important to study the involvement of sensory nervous system. Few authors have described afferent nerve fibers expressing TRPV1 receptors among the extrinsic sensory neurons in rodents (Patterson et al. 2003; Ward et al. 2003), while others have found TRPV1-like immunoreactivity on intrinsic enteric neurons in guinea pigs, domestic pigs and human (Poonyachoti et al. 2002; Anavi-Goffer & Coutts 2003; Chan et al. 2003). Under physiological circumstances, the TRPV1-expressing afferent neurons play important role in the regulation of gastrointestinal circulation, secretion, mucosal homeostasis, motility and nociception (Holzer & Barthó 1996; Holzer & Maggi 1998).

The adequate pharmacological treatment of inflammatory bowel diseases is still a major problem nowadays. Treatments come with several unwanted sideeffects. As a result of latest developments, specific antibodies against proinflammatory mediators had been introduced into therapy (anti-TNF- α antibody, infliximab), but the underlying pro- and antiinflammatory regulatory mechanisms are still not fully elucidated.

The most likely cause of inflammatory bowel disease is the damage of the interepithelial tight junctions and the reaction triggered by the invading luminal antigens. One important component of this process is the activation of the sensory nerve endings and neurogenic inflammation (Yamamoto et al. 1996; Gross & Pothoulakis 2007). There is a novel finding that the SP released from TRPV1 expressing neurons is an aggravating factor in ulcerative colitis (Engel et al. 2012).

1.6 Necessity of the development of new drugs

Currently the adequate therapy of inflammatory disease is not resolved as specific inhibitor of the neurogenic component of inflammation is not available. One of this significant health problem is the group of inflammatory bowel diseases and the airway inflammations. The role of neurogenic component has been proved to be relevant in the development of inflammation. I focused on the inflammation of bowels and airways as both are big organ systems, direct connection with the external environment, their inflammation is affecting numerous people.

As the exact pathomechanism is not fully uncovered (and probably we are facing with multifactorial disease), causal therapy cannot be performed, the aim is the control of the disease and deceleration of its runoff (Gaál 2012).

There are several high efficiency antiinflammatory agents in the treatment of bowel inflammations (corticosteroids, TNF- α inhibitors), but they can cause severe sideeffects especially during chronic administration. Currently, the substances be classified in three main groups: local corticosteroids, systemic immunosuppressants (methotrexate, cyclophosphamide and azathioprine) and the newest monoclonal antibodies (infliximab, etanercept, adalimumab, certolizumab, natalizumab).

Similar approach is used in the treatment of airway inflammations. The therapy of chronic airway inflammatory diseases (asthma bronchiale) is based on locally administered corticosteroids (budesonid, fluticason, mometason), and the systemic administered leukotriene antagonists (montelukast, zafirlukast, pranlukast). These drugs should be administered continuously to prevent the development of chronic inflammation which can lead to acute exacerbations. In severe cases, biologic therapy is used (omalizumab in the corticosteroid resistant allergic asthma).

Opportunistic infections (fungal, bacterial and viral infection) may arise as a consequence of local or systemic immunosuppression or endocrine changes can occur due to the disturbance of hormonal balance. Also, the newest biologic treatments are not free from sideeffects; exacerbation of severe, latent infections (tuberculosis) or development of multiple sclerosis, progressive multifocal leukoencephalopathy (PML) were observed (Mir Subías et al. 2013; Piusińska-Macoch 2013). Furthermore, the biologic therapy is expensive.

The chronic inflammation may cause irreversible damage in the lung, the permanent tissue remodeling (emphysema, fibrosis) interferes with the pulmonary functions and lowers the

quality of life of the patient. Obviously, surgical intervention is not available which could improve the condition of the patient.

All of these justify to make efforts to develop anti-inflammatory agents with new mechanism by clarifying the regulatory mechanisms of inflammation. One of the possible method could be the use of selective receptor antagonism to intervene the development of inflammation.

2. Aims

Airway inflammation model:

- a) Investigating the role of the neurokinin 1 receptor in the LPS-induced airway inflammation.
- b) Explore the role of certain transmitters (SP, NKA) and their receptor (NK1) in the endotoxin-induced airway inflammation, analyzing the effects of mediators and their receptors to the different components of inflammatory processes.
- c) Observe the inflammatory processes and their consequences (airway hyperreactivity) caused by chronic cigarette smoke exposure in Trpv1 KO and Tac1 KO, NK1 KO mice.

Bowel inflammation model:

- a) Analyze the role of the TRPV1 receptors in dextran-sulfate induced colitis ulcerosa in animal model with two DSS concentrations.
- b) Investigate the expression of NK1 receptors and different tachykinin genes in intact and colitic animals.
- c) Discover the effect of NK1 receptor antagonism in DSS-induced colitis on local cytokine production.

3. Experimental models and methods

3.1 Airway inflammation models

3.1.1 Animals

All of the animals used in this experiment came from C57Bl/6 strain or was genetically engineered based on this strain. The mature mice weighed 20-25 g and were 8-10 week old. Both gender was used in both group equally.

The production of Tac1 KO (Zimmer et al. 1998) and NK1 KO (Tacr1 KO) mice was described in details previously (De Felipe et al. 1998; Laird et al. 2000). The original breeding pair of the

double knockout strain was generated with the selective crossbreeding of these strains (Tac1 KO x NK1 receptor KO) and these mice lack the SP/NK1 signal transduction pathway entirely.

3.1.2 Induction of airway inflammation and experimental setup

3.1.2.1 Subacute airway inflammation

The subacute airway inflammation was evoked by intranasal instillation of 60 μ l *Escherichia coli* (serotype: O83) endotoxin (167 μ g/ml dissolved in sterile PBS) and measurements were taken after 24 hours. Control animals received equal volume of sterile PBS.

3.1.2.2 Chronic airway inflammation

Chronic airway inflammation was induced by two-month whole body smoke exposure. Animals were placed in cages into the smoke exposure chamber and they received smoke exposures 10 times a week. Reference cigarette was used for this purpose (Reference Cigarette, type 3R4F).

3.1.3 Measurement of airway reactivity

Conscious, freely breathing animals were measured by whole body plethysmography. Nebulized saline and then muscarin receptor agonist carbachol in increasing concentrations (50 μ l per mouse in 0, 5.5, 11, 22 mM concentration) was sprayed into the chamber to evoke bronchoconstriction after 24 hours of LPS instillation. The so-called "enhanced pause" (P_{enh}) was calculated as the indicator of airway resistance. Similar method was followed during the measurement of airway inflammation during the smoke exposure experiment. 0, 11, 22 mM concentration of carbachol was nebulized into the chambers according to previous experiments. Airway reactivity was assessed monthly.

3.1.4 Bronchoalveolar lavage

Mice were anaesthetized with ketamine-xylazin and their lungs were washed out with 5 ml of cold PBS solution in five portions via a tracheal cannula. The lavage fluids were weighed and the cells stained with CD45 FITC and propidium iodide were counted Partec flow cytometer device.

3.1.5 Histopathological analysis and scoring

The whole right lobe of lung was removed and fixated in 4% formaldehyde for 8 hours, after embedding in paraffine, 5-7 μ m thick sections were made with microtome and stained with hematoxylin-eosin routinely. Mucus producing goblet cells were visualized with periodic acid

Schiff reaction. Sections were evaluated by an expert pathologist in a blinded fashion (Zeldin et al. 2001) semiquantitatively, and composite inflammatory score was calculated.

3.1.6 Assessment of myeloperoxidase (MPO) activity

The spectrophotometric MPO assay was performed from the thawed samples on a 96-well plate. Briefly, 25 μ l assay buffer, 25 μ l samples were pipetted in triplicates onto the plate, then 100 μ l H_2O_2 -3,3',5,5'-tetramethyl-benzidine (TMB/ H_2O_2) was pipetted into each well. Absorbances were read two times: first immediately after the TMB solution was added and second time after 5 minutes (kinetic reaction) at 620 nm wavelength on a Multiskan plate reader. MPO activity was calculated from the absorbance changes with a calibration curve. MPO activities were expressed in unit/mg wet tissue.

3.1.7 Measurement of inflammatory cytokines in the lung

Homogenization of lung tissue samples were done in 450 μ l RPMI 1640 buffer which contained 50 μ l phenyl-methyl-sulphonyl-fluoride (PMSF). The supernatant of centrifuged homogenates was used for the measurement of interleukin 1 β (IL-1 β) and tumor necrosis factor- α (TNF- α) concentration with specific ELISA technique.

3.1.8 IL-1 β cytokine measurement

For the measurement of IL-1 β cytokine concentration, the thawed tissue samples were weighed and homogenized in 0.5 ml RPMI medium containing 1% PMSF on ice for 1 minute. Then the homogenates were centrifuged in 4 $^\circ\text{C}$ with 10000 g acceleration for 10 minutes and the supernatants were used according to the manufacturer's directions. Results were presented in ng/g tissue.

3.1.9 Statistic

The percentage P_{enh} values justified to the baseline, the MPO activities and cytokine concentrations were expressed as mean \pm SEM and evaluated with two-way ANOVA followed by Newman-Keuls posttest. The histopathological scores were assessed with Kruskal-Wallis test followed by Dunn posttest. Statistical analysis was made with GraphPad Prism 5.02 for Windows (GraphPad Software, USA). Probability values < 0.05 were accepted as significant.

3.1.10 Ethics

All experimental procedures were carried out 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 University of Pécs according to the Ethical Codex of Animal Experiments and licence was given (licence number: BA BA 02/2000-11-2006).

3.2 Bowel inflammation models

3.2.1 Animals

Female Trpv1 gene-deleted (knockout, KO) mice and their C57Bl/6 wild-type counterparts (8–10 weeks old, 20–25 g) were used for this study. The original breeding pairs of both types were purchased from Jackson Laboratories (USA) through Charles-River Hungary.

In the experiment focusing on NK1 receptors and tachykinins, male NK1 receptor (Tacr1 KO) gene-deficient mice were used and backcrossed for 8-10 generations to C57B/6 mice. C57B/6 mice were used as wild type (WT) controls. NK1 KO mice were generated at the University of Liverpool as previously described (Zimmer et al. 1998; De Felipe et al. 1998; Laird et al. 2000; Helyes et al. 2004).

3.2.2 Experimental setup

3.2.2.1 Induction of colitis

Colitis was induced with 2% and 5% DSS dissolved in the drinking water and administered for 7 days. The intact control group of animals received only tap water. Then mice were sacrificed in deep ketamine-xylazine anaesthesia (100 mg/kg ketamine i.p.; 5 mg/kg xylazine i.p.) after fasting overnight. The distal colon samples (one third of the colon from anus to cecum) were dissected to determine receptor expression, myeloperoxidase enzyme activity, cytokine measurement and histopathological evaluation (Szitter et al. 2010).

3.2.2.2 Netupitant treatment

The selective synthetic NK1 receptor antagonist netupitant was administered i.p. (6 mg/kg, 100 µl from the 0.6 mg/ml solution) once a day from the beginning of the experiment for a total duration of one week. Mice in the control group of the DSS treatment received the vehicle in the same volume according to the same treatment paradigm. A group of receptor deficient mice also received the netupitant treatment to identifying any effect caused by netupitant beside NK1 receptor blockade. Netupitant stock solution was prepared according to the instruction of the manufacturer. Stock solution was stored in 2 °C. This stock solution was diluted in saline to prepare the final 0.6 mg/ml solution freshly every day.

3.2.3 Receptor expression

Colon samples were stored in RNAlater and homogenized in 1 ml of TRI Reagent. Isolation of total RNA was carried out according to the manufacturer's protocol. The obtained cDNA samples were amplified with PCR using specific primers. β -actin served as the reference housekeeping gene. PCR products were run on agarose gels containing DNA specific dye and visualized under UV light.

3.2.4 Disease Activity Index (DAI) assessment

The clinical symptoms of colitis, such as body weight change, stool consistency and fecal blood content, were scored on a daily basis. Fecal blood content was assessed with Hemocare test which uses a modified guaiac method. Scores for the 3 parameters were averaged for each mouse to obtain the Disease Activity Index (Kihara et al. 2003).

3.2.5 Histological evaluation

The distal colon samples were fixed in 4% buffered formaldehyde, embedded in paraffin, sectioned (5 μ m), and stained with haematoxylin and eosin. Digital micrographs were taken. Inflammatory alterations were evaluated and scored by an expert pathologist blinded from the experimental design (Kihara et al. 2003). Additionally, quantitative assessment was also performed to evaluate the histopathological severity of inflammation: the number of inflamed foci was counted and percentage of the damaged area was calculated on the digital micrographs.

3.2.6 Myeloperoxidase measurement

The same method was used as in 3.1.6 section.

3.2.7 Measurement of IL-1 β concentration

The similar method was applied as described in the 3.1.8 section.

3.2.8 Cytokine panel assay

The cytokine assay was performed according to the manufacturer's instructions. Briefly, the excised and frozen tissues were thawed and weighed, and immediately placed in PBS containing 10 mg/ml phenyl methyl sulfonyl fluoride (PMSF) protease inhibitor, and homogenized as described above. Then Triton X-100 was added to the samples to a final concentration of 10 mg/ml and centrifuged at 10000g for 5 minutes to remove cell debris. Total protein concentrations were determined prior to cytokine measurement. Chemiluminescent detection was performed. The intensity of the emitted light at each spot was

proportional to the amount of bound cytokine. Results were calculated by densitometry using ImageJ freeware.

3.2.9 Statistics

Results are expressed as means \pm SEM. For the evaluation of the data, two-way ANOVA (DAI) followed by Bonferroni's post-test; unpaired t-test with Welch correction (MPO activity, cytokine profile, quantitative histopathological data) or Mann-Whitney U-test (semiquantitative histopathological scores) was used. Statistical analysis was done using GraphPad Prism 5.02 for Windows (GraphPad Software, USA). Probability values < 0.05 were accepted as significant.

3.2.10 Ethics

All experimental procedures were carried out 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 University of Pécs according to the Ethical Codex of Animal Experiments and licence was given (licence number: BA 02/2000-2/2012).

4. Results

4.1 Airway inflammation model

4.1.1 Investigation of the role of tachykinins and NK1 receptors in the endotoxin-induced acute airway inflammation

4.1.1.1 Inflammatory airway hyperreactivity in Tac1, NK1 and double knockout mice

Baseline P_{enh} significantly increased 24 h after intranasal LPS treatment in all groups compared to the respective PBS-treated control mice. Inhalation of increasing concentrations (5.5, 11 and 22 mM) of the muscarinic receptor agonist carbachol evoked a concentration-dependent bronchoconstriction. In wild type mice responses demonstrated as percentage increase of P_{enh} above baseline were markedly enhanced in the LPS-treated group compared to the respective non-inflamed controls which supports the development of inflammatory bronchial hyper-responsiveness. In Tac1 and Tac1/NK1 gene-deficient mice the LPS-induced airway hyper-reactivity was markedly reduced, particularly at the highest carbachol concentration. LPS-evoked airway hyper-reactivity was not significantly decreased in the NK1 receptor-deleted group.

4.1.1.2 Inflammatory histopathological changes in the lung of Tac1, NK1 and double knockout mice
LPS induced marked peribronchial/perivascular oedema formation, neutrophil accumulation around the bronchi, infiltration of activated recruited macrophages/lymphocytes into the alveolar spaces and moderately increased number of mucus producing goblet cells in the wild type group. In the lung of PBS-treated control mice there were no inflammatory changes, only some macrophages could be observed in the alveolar spaces. Meanwhile, in mice lacking SP and NKA due to the deletion of the Tac1 gene, the extent of the oedema and the number of the oedematous structures, neutrophil accumulation as well as macrophage infiltration, but not goblet cell hyperplasia were significantly less intense, therefore the composite inflammation scores calculated from these parameters were also markedly lower. In contrast, these inflammatory parameters were not altered by NK1 receptor deletion. Surprisingly, a lower oedema intensity and neutrophil accumulation observed in case of missing SP and NKA was not seen in mice that did not express the NK1 receptor. We have not observed any changes in the control mice in any of the three knockout groups.

4.1.1.3 Myeloperoxidase activity in the lung homogenates

LPS induced more than 2-fold elevation of MPO activity in the lung 1 day after intranasal administration. This quantitative marker of accumulated neutrophils and macrophages in the inflamed tissue was significantly decreased in Tac1 gene-deficient mice, but not in the NK1 and the Tac1/NK1 knockout animals.

4.1.1.4 Inflammatory cytokine concentrations in the lung

Lung IL-1 β and TNF- α levels markedly increased 25 h after intranasal LPS administration compared to the concentrations measured in the lungs of PBS-treated control animals. The absolute concentration of the latter inflammatory cytokine was about 10- fold less. Lung TNF- α , but not IL-1 β concentration was significantly lower in the Tac1 KO group. In contrast, LPS-induced IL-1 β and TNF- α production was elevated in NK1 receptor-deficient animals. No change was observed, however, in cytokine concentrations in the pulmonary tissues of the double knockout mice

4.1.2 The role of the TRPV1, NK1 receptors and Tac1 gene products tachykinins in cigarette smoke induced chronic airway inflammation

4.1.2.1 Airway reactivity

There is a significant elevation of airway reactivity in the Trpv1 KO mice from the fourth week. The NK1 receptor deficient and Tac1 KO animals show increasing tendency only from the 8th week but the difference is not significant.

4.1.2.2 Histopathological changes in chronic airway inflammation

The appearance of granulocytes and macrophages can be noticed on the slides caused by the cigarette smoke evoked chronic inflammation. The inflammatory cell accumulation is more prominent in the Trpv1 KO animals especially from the second month. This changes was less marked in Tac1 KO mice compared to C57Bl/6 animals while the NK1 KO animals showed more marked edema formation and macrophage infiltration compared to the wild type mice. These results underline the results of BALF analysis.

4.1.2.3 Cell count in bronchoalveolar lavage fluid

The number of granulocytes was elevated in the first month in the C57Bl/6 mice compared to the non smoking counterparts and smoking Trpv1 KO animals produced statistically more macrophages compared to the smoking wild type animals. At the end of second month, all of the inflammatory cell numbers were elevated markedly in the Trpv1 KO animals both in the smoking and non-smoking group compared to the wild type mice. The absence of the NK1 receptors leads to less but significantly elevated cell count compared to the wild type smoking groups of animals.

4.1.2.4 Myeloperoxidase enzyme activity

All smoking group showed significantly higher myeloperoxidase enzyme activity at the end of the first month compared to the intact animals. At the end of second month the MPO enzyme activity was significantly higher in the Trpv1 KO and NK1 KO animals compared to the wild type smoking animals.

4.1.2.5 IL-1 β cytokine production

The level of IL-1 β cytokine increased in the Trpv1 KO mice after 1 and 2 month of smoking. There was no significant changes in the Tac1 KO and NK1 KO animals compared to the wild type mice.

4.2 Bowel inflammation models:

4.2.1 The effect of TRPV1 receptors in dextran-sulfate induced murine colitis

4.2.1.1 Disease Activity Index

By the end of the seventh day, the clinical symptoms of colitis developed in both the 2% and 5% DSS-treated groups. Genetic lack of the TRPV1 receptor significantly decreased the DAI in the 2% DSS-treated group after day 5. This difference could not be observed in the 5% DSS-treated group, where severe bloody diarrhea developed both in wild type and knockout animals. Five percent DSS produced significantly higher DAI compared to 2% DSS treatment. The composite scores were elevated mainly by the increasing blood content of stool.

4.2.1.2 Semiquantitative histopathological analysis and scoring

Histopathological scores were evaluated separately for each segment of the colons. Composite inflammation scores were higher in all the DSS-treated mice compared to the tap water treated control animals in which no signs of inflammation were seen. Treatment with 5% DSS produced significantly higher tissue damage than the 2% concentration. In the 2% DSS-treated group, the genetic lack of TRPV1 receptors attenuated the histological changes: there was a tendency of milder inflammatory signs; indeed, it was statistically significant in the distal segments. In the 5% DSS-treated group, the absence of TRPV1 receptors did not decrease but even showed a worsening tendency on the histopathological parameters.

*4.2.1.3 Myeloperoxidase enzyme activity in *Trpv1* Ko and wild type mice*

Oral DSS application for 7 days, both in 2% and 5% concentrations, markedly increased MPO activity in all the three colon segments compared to the control mice drinking tap water. MPO elevation induced by the 2% DSS was significantly higher in all the samples of C57Bl/6 mice than in the respective tissues of *Trpv1* KO mice. Although 5% DSS treatment caused substantial increase of myeloperoxidase content of colon samples compared with the tap water-treated controls, there were no significant differences between wild-type and KO animals.

*4.2.1.4 IL-1 β production in *Trpv1* Ko and wild type animals*

Two percent DSS-induced IL-1 β production was only considerable in the distal colon segments; the concentrations of this cytokine in the other colon segments were similar to that measured in the intact tissues. This moderate, non-significant IL-1 β elevation in response to 2% DSS was similar in the *Trpv1* KO and wild type mice. In contrast, 5% DSS administration caused

significant drop in IL-1 β levels in both groups; the concentrations were much lower than those measured in the homogenates of the intact colons.

4.2.2 The effect of neurokinin 1 receptor and selective receptor antagonist (netupitant) and the tachykinin system in the dextran-sulfate induced colitis model.

4.2.2.1 Receptor expression

The receptor expression study revealed that the Tac1 gene (encoding Substance P and NKA) was expressed in the distal colon both in the intact control and DSS-treated mice. We could not detect the Tac3 and Tac4 genes of NKB and HK-1, respectively. Inflammation did not alter this expression pattern. The Tacr1 gene expressing the NK1 tachykinin receptor was significantly up-regulated after 7 days of oral DSS administration. There was no expression of the Tacr2 and Tacr3 genes of the NK2 and NK3 tachykinin receptors in the intact colon, but they were moderately or minimally up-regulated in response to DSS administration.

4.2.2.2 Disease Activity Index

DAI was calculated daily on the basis of body weight, stool consistency and fecal blood content was significantly reduced from day 6 both by genetic deletion and selective antagonism of the NK1 tachykinin receptor with 6 mg/kg i.p. netupitant administration. Netupitant-treated NK1 receptor-deficient mice showed similar Disease Activity Index compared to vehicle-treated receptor-deficient animals.

4.2.2.3 Histological analysis

Compared to a histopathological picture of the non-inflamed colon structure showing intact crypts and normal mucosal epithelial layer, 2% DSS drinking resulted in a remarkable inflammation and tissue damage in the C57Bl/6 wild type group without netupitant treatment. There was a significant mucosal and submucosal neutrophil infiltration, loss of the crypts and disintegration of the mucosal structure. The severity and the extent of these characteristic histopathological alterations were reduced by both NK1 receptor deletion and daily administration of the NK1 receptor antagonist netupitant. Meanwhile, in the NK1 KO animals netupitant treatment did not influence the severity of inflammation compared to the vehicle-treated group. These findings were confirmed by the significantly diminished semiquantitative score values determined in the NK1 receptor-deficient and netupitant-treated groups in comparison with the solvent-treated wild type animals. The quantitative results describing the

number of inflamed foci and the percentage values of the damaged areas also showed an anti-inflammatory effect of netupitant.

4.2.2.4 Myeloperoxidase enzyme activity

DSS administration induced an approximately 2-fold increase in MPO activity of the colon homogenates compared to the respective intact samples of water-drinking wild type and NK1 receptor-deficient mice. This elevation was significantly decreased by 6 mg/kg i.p. daily netupitant treatment. Surprisingly, netupitant decreased the MPO activity in DSS-treated NK1 receptor-deficient mice as well compared to their vehicle-treated controls similarly to what was observed in wild types.

4.2.2.5 Murine inflammatory cytokine chip (cytokine array panel)

Among the 40 investigated cytokines, 11 (BLC, sICAM-1, IFN- γ , IL-1 α , IL-1ra, IL-13, IL-16, IP-10, JE, MIG, and TIMP-1) were significantly increased in the colon homogenates in response to DSS administration in wild type mice compared to their intact controls. In contrast, in the NK1 receptor-deficient group BLC, sICAM-1, IFN- γ , IL-1 α , IL-16, JE did not increase significantly while IL-1ra, IP-10, MIG and TIMP were similarly elevated in wild type animals. There was no difference between the expressions of these cytokines in the intact colon samples of the two groups. Netupitant treatment significantly decreased the DSS-induced elevation of BLC, IFN- γ , IL-13 and IL-16 levels compared to vehicle administration. These changes were similar to the results found in NK1 receptor-deficient mice.

5. Summary of new results, conclusions

1. In the absence of Tac1 encoded tachykinins (SP and NKA) – while not in the absence of NK1 receptor – the endotoxin-induced bronchial hyperreactivity, MPO production and inflammatory cytokine release are decreased. These peptides are important effectors in the acute lung inflammation model but not via the activation of NK1 receptors.
2. Every inflammatory parameter is aggravated in the smoke-induced airway inflammation model in the absence of TRPV1 receptor which means that the activation of this ion channel has protective effect. In the lack of SP/NK1 and NK1 receptor increased MPO activity was observed which can be explained by the increased macrophage infiltration according to the histological analysis and BALF results. In contrast, the IL-1 β production did not change. Surprisingly, in this chronic model (but not in the acute lung inflammation) the Tac1 encoded tachykinins inhibit certain inflammatory parameters via the activation of NK1 receptors.
3. The ideal concentration of DSS is 2% in our experiments which with colitis is rendered good in mice. Higher concentration such as 5% is proved to be cytotoxic, the tissue destruction makes it inappropriate to study the disease. The TRPV1 receptors play proinflammatory role in the DSS induced murine colitis ulcerosa model as the developing inflammation was less aggravated considering the physiological, histological and immunological results.
4. The expression of Tac1 gene is not upregulated in the DSS induced colitis model but the expression of NK1 receptors is increasing. Significantly less inflammatory changes were observed on functional, histological and molecular level in case of receptor antagonism or receptor deficiency. The NK1 receptor regulates inflammatory processes in the gut in a complex manner because the lack of NK1 receptor results in increased MPO activity. Results from cytokine panel assay suggest that the activating NK1 receptors increase B-cell migration, sICAM-1 enhances the adhesion and transmigration of different immune cells. The inhibition of NK1 receptors decreases the production of BLC, IFN- γ , IL-13, IL-16 cytokines therefore they might be promising new targets of further pharmacological investigation.

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List of publications

Full-length articles related to the present thesis

Szitter I, Pintér E, Perkecz A, Kemény Á, Kun J, Pietra C, Quinn J P, Zimmer A, Berger A, Paige C J, Helyes Zs „Role of neurokinin 1 receptors in dextran sulfate-induced colitis: studies with gene-deleted mice and the selective receptor antagonist netupitant”. *Inflammation Research* (2014) (IF: 2,143 (2013))

Szitter I, Pozsgai G, Sándor K, Elekes K, Kemény Á, Perkecz A, Szolcsányi J, Helyes Zs, Pintér E “The role of transient receptor potential vanilloid 1 (Trpv1) receptors in dextran sulfate-induced colitis in mice”. *Journal Of Molecular Neuroscience* 42:(1) pp. 80-88. (2010) (IF: 2,922, FC: 12)

Helyes Zs, Elekes K, Sándor K, **Szitter I**, Kereskai L, Pintér E, Kemény A, Szolcsányi J, McLaughlin L, Vasiliou S, Kipar A, Zimmer A, Hunt S P, Stewart J P, Quinn J P “Involvement of preprotachykinin A gene-encoded peptides and the neurokinin 1 receptor in endotoxin-induced murine airway inflammation”. *Neuropeptides* 44:(5) pp. 399-406. (2010) (IF: 1,917 FC: 7)

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Conference presentations related to the present thesis

Szitter I, Pintér E, Perkecz A, Kemény Á, Pietra C, Quinn J P, Zimmer A, Berger A, Helyes Zs „Effect of neurokinin 1 (NK1) receptor antagonism and the differential role of NK1 receptors in dextran-sulfate (DSS) induced colitis in mice” In: Proceedings of the British Pharmacological Society. Place and date of conference: Granada, Spain, 2012.07.17-2012.07.20. Paper C049 (2012) (poster)

Szitter I, Pintér E, Kemény Á, Pietra C, Quinn J, Zimmer A, Berger A, Helyes Zs “Neurokinin 1 (NK1) receptor antagonizmus hatása és a NK1 receptorok szerepe a dextrán-szulfáttal (DSS) kiváltott colitisben egérmodellben” p. 189. A Magyar Élettani Társaság a Magyar Anatómusok Társasága a Magyar Biofizikai Társaság és a Magyar Mikrocirkulációs és Vaszkuláris Biológiai Társaság Kongresszusa abstract booklet, Debrecen, Hungary, 2012.06.10-2012.06.13 (2012) (poster)

Szitter I, Pintér E, Perkecz A, Berger J A, Paige C J, Szolcsányi J, Helyes Zs „Role of the tachykinin system in dextran sulphate-induced colitis in mice” In: *Journal of Molecular Neuroscience*. Place and date of conference: Boston, USA, 2011.05.22-2011.05.25. p. 199. (2011) (poster)

Szitter I, Pintér E, Pozsgai G, Sándor K, Kemény Á, Perkecz A, Elekes K, Helyes Zs, Szolcsányi J, Quinn J “Investigation of the role of TRPV1 capsaicin receptors and tachykinins in dextrane-sulphate induced mouse colitis model” Tue 271. 16th World Congress on Basic and Clinical Pharmacology (WorldPharma2010), Copenhagen, Denmark 2010.07.17-2010.07.23. (2010) (poster)

Szitter I, Pintér E, Sándor K, Bánvölgyi A, Elekes K, Szolcsányi J, Quinn J, Helyes Zs “Investigation of the role of TRPV1 capsaicin receptors and tachykinins in dextrane-sulphate induced mouse colitis model” *Acta Physiologica Hungarica* 97:(1) pp. 139-140. (2010) (IF: 1,226) (poster)

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Sütő B, **Szitter I**, Bagoly T, Pintér E, Szolcsányi J, Loibl Cs, Námeth T, Tánczos K, Molnár T, Leiner T, Várnai B, Bardonicsek Zs, Helyes Zs “Plasma somatostatin-like immunoreactivity increases in the plasma of septic patients and rats with systemic inflammatory reaction: experimental evidence for its sensory origin and protective role”. *Peptides* 54: pp. 49-57. (2014) (IF: **2,614 (2013)**)

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Kun J, Helyes Zs, **Szitter I**, Gódi Sz, Szabó I, Kemény Á, Pohóczky K, Perkecz A, Pintér E “A tranziens receptor potenciál ankyrin 1 (TRPA1) és vanilloid 1 (TRPV1) receptor mRNS expresszió gyulladós bélbetegségben (IBD) és dextrán-szulfát (DSS) által indukált egér colitis modellben” p. A-0184. A Magyar Élettani, Farmakológiai és Mikrocirkulációs Társaságok 2013. évi közös Tudományos Kongresszusa, Budapest, Hungary, 2013.06.05-2013.06.08. (2013) (poster)

Szitter I, Barbara K, Kiss T, Perkecz A, Feller D, Helyes Zs “Tüdő morfometria: dohányfüst okozta szöveti elváltozások kvantitatív követése mikroCT módszerrel C57Bl/6 és galanin 3 receptor (Gal3r) deficiens egerekben” p. A-0191. A Magyar Élettani, Farmakológiai és Mikrocirkulációs Társaságok 2013. évi közös Tudományos Kongresszusa, Budapest, Hungary, 2013.06.05-2013.06.08. (2013) (poster)

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Helyes Zs, **Szitter I**, Hajna Zs, Elekes K, Kemény Á, Kereskai L, Quinn J P, Sándor K, Pintér E, Szolcsányi J
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Kun J, Pintér E, **Szitter I**, Szabó I, Gódi Sz, Perkecz A, Helyes Zs “A tranziens receptor potenciál ankyrin 1 (TRPA1) receptor expresszióváltozása gyulladásoos bélbetegségben (IBD) és dextrán-szulfát által indukált egér colitis modellben” p. 135. A Magyar Élettani Társaság a Magyar Anatómusok Társasága a Magyar Biofizikai Társaság és a Magyar Mikrocirkulációs és Vaszkuláris Biológiai Társaság Kongresszusa abstract booklet, Debrecen, Hungary, 2012.06.10-2012.06.13. (2012) (poster)

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Elekes K, Sándor K, Szőke E, Tóth D, Markovics A, **Szitter I**, Jakab L, Szakacs B, Kereskai L, Szolcsányi J, Molnár T, Helyes Zs “Peripheral effect of the marijuana smoke in the lung of mice” Mon 283. 16th World Congress on Basic and Clinical Pharmacology (WorldPharma2010), Copenhagen, Denmark, 2010.07.17-2010.07.23. (2010) (poster)

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