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Changes in the expression of galanin and galanin receptors in the wall of the colon in pigs experimentally infected with *Brachyspira hyodysenteriae*

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Abstract

The expression of galanin (GAL) and its three receptors (GalR1, GalR2, and GalR3) were studied with real-time PCR in the colonic wall of pigs suffering from experimental colitis caused by the infection with *Brachyspira hyodysenteriae*. The expression was studied in the muscular membrane, mucosa/submucosa layer, and in lymphocytes isolated from mucosa/submucosa. The expression levels were normalized to glyceraldehyde-6-phosphate dehydrogenase (GAPDH) expression and compared to expression levels in control animals. GAL expression was found in all three studied compartments of the colonic wall. A significant decrease in GAL expression level was found in the mucosa/submucosa and in isolated lymphocytes, whereas the decrease was much less profound in the muscular membrane. In the case of galanin receptors their expression was found in all studied compartments of the colonic wall, however at different levels, as compared to GAPDH expression. The decrease of galanin receptors expression was found in all studied compartments of the colonic wall of the sick animals.

Key words: pig, colitis, galanin, galanin receptors.

Introduction

Ulcerative colitis (UC) and Crohn's disease (CD) are highly debilitating diseases affecting thousands of people around the world, mainly in developed countries (12). Up till now, our understanding of the pathogenesis of these diseases is quite limited. Some aspects of UC and CD are studied using samples derived from human biopsies or intestines removed due to intractable, life-threatening pathological changes (6). However, researchers need effective and reliable animal models to study the mechanism of development of intestinal inflammation. Rodent models (rat and mouse) are commonly used, in which different agents are used to induce the enteritis. However, data obtained in rodents cannot directly reflect processes occurring during human enteritis, thus other animal models are required to improve our knowledge on these processes. We developed an animal model for studies on enteritis using experimental infection of the domestic pig with *Brachyspira hyodysenteriae*, a Spirochaetal bacterium evoking necrohaemorrhagic bowel inflammation (8).

Many factors are involved in the development, course, and descent of the inflammation. Neuropeptides, being short peptides acting as neurotransmitters, cotransmitters, and neuromodulators in the nervous system, are also involved in the inflammatory process. One, most commonly known, is the substance P released by sensory nerve terminals and responsible for neurogenic inflammation. Other neuropeptides involved in the inflammatory process are neurokinines A and B, pituitary adenylate cyclase-activating peptide (PACAP), vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP), somatostatin (SOM),

neuropeptide Y (NPY), and galanin (GAL). Changes in the expression of neuropeptides were found in the wall of the gut in Crohn's disease and ulcerative colitis in humans (6). We conducted studies on the changes in the concentrations of neuropeptides and changes in neuropeptide-containing nerve structures in the intestinal wall, autonomic ganglia innervating the intestines, and in intestinal lymph nodes during the swine dysentery induced by an experimental infection with *B. hyodysenteriae*. Studies with ELISA indicated that the tissue concentrations of VIP, SOM, NPY, and SP changed mainly in the inferior mesenteric ganglion (IMG) and coeliac-superior mesenteric ganglion (CSMG), two autonomic ganglia responsible for innervation of the small and large intestines (9). GAL was the neuropeptide, which concentration changed in the intestinal wall. GAL is a neuropeptide involved in many physiological and pathological processes (10). It is responsible for regulation of the intestinal motility and for the regulation of ionic transport across the mucosa, thus regulating the water resorption in the colon (1, 3, 15). It is strongly associated with the inflammatory processes, and it is connected with skin inflammation and arthritis (2, 11). It is also involved in the transmission of inflammatory pain. GAL is expressed not only by nerve cells present in the intestinal wall, but may be expressed by certain classes of leukocytes (21).

GAL exerts its action on the peripheral tissues through three known receptors belonging to the class of G protein coupled transmembrane receptors: GalR1, GalR2, and GalR3 (23).

GalR1 is widely expressed in the peripheral tissues, among others in the intestines. It inhibits the adenylate cyclase lowering the level of cAMP. GalR2 receptor acts *via* Gq/phospholipase C/protein kinase C pathway, although it was also shown to activate G protein. It is distributed in the central nervous system and peripheral tissues. The third receptor, GalR3, is also distributed both centrally and peripherally. Expression studies have shown the expression of the three receptors in many types of cells, including the cells of the immunological system (21).

We decided to perform the study on changes in the expression of GAL and its three receptors at the level of mRNA in the wall of the colon in pigs experimentally infected with *B. hyodysenteriae*. However, to understand better the changes, the expression of the chosen transcripts was studied in three compartments of the colon wall: mucosa and submucosa, muscular membrane, as well as leukocytes isolated from the mucosa.

Material and Methods

The study was performed on two groups of female pigs of the Large White Polish breed: group of clinically healthy animals (n = 4) and experimental group (n = 4). The study was accepted by the Local Ethical Committee of the University of Warmia and Mazury in Olsztyn, Poland. The procedures of the experimental infection with *B. hyodysenteriae* and sacrifice were described elsewhere (9). Shortly, the animals were inoculated *per os* with a *B. hyodysenteriae* reference strain B204 (ATCC 31212) obtained from the National Veterinary Research Institute in Pulawy, Poland. The bacteria were grown on an appropriate solid medium under anaerobic conditions. Animals were inoculated *via* a gastric tube with bacterial colonies scraped from two Petri dishes and suspended in a phosphate buffered saline (PBS). After approx. one week, the first symptoms of enteritis developed (lack of appetite, loose stools, fever). After additional 5-7 d, the full clinical picture of dysentery developed (strong, dysenteric diarrhoea with flecks of mucus, fragments of mucosa, and blood). The animals were weak, emaciated, and dehydrated. At this stage, the animals were sacrificed and *post mortem* examination was performed. The mucosa was thickened, ulcerated, and covered with fibrin. Fragments of the colonic wall were subjected to histopathological examination, which revealed microscopic changes typical for swine dysentery.

To obtain total RNA preparations from the mucosa and tunica muscularis, fragments of the descending colon wall from control and experimental animals were collected and placed into RNALater® (Ambion, USA). Then the mucosa was scraped and the total RNA was isolated from the mucosa and tunica muscularis with a Total RNA Mini Plus kit (A&A Biotechnology, Poland).

To obtain total RNA from mucosal leukocytes, the fragment of the colonic wall was dissected out and the mucosa was gently scraped with a scalpel. The scraped tissue was placed in 100 mL of Hank's buffered salt solution containing 8 mM dithiotreitol (DTT) and incubated at 37 °C for 45 min with a gentle stirring. Then, the tissue suspension was filtered through the Perlon wool and the filtrate was centrifuged for 15 min at 3000 rpm (Hereus Megafuge 16R, Thermo Scientific, Lithuania). The resulting pellet was suspended in 20 mL of a Leibowitz solution and the suspension was overlayed on the Gradisol L solution (Aqua-Med, Poland) in multiple tubes (5 mL of Gradisol L and 10 mL of cell suspension). Then the tubes were centrifuged for 15 min at 400 g. The interfase containing the lymphocytes was carefully collected and centrifuged for 2 min at 7000 rpm (Hereus Megafuge 16R, Thermo Scientific, Lithuania). The resulting pellet was washed with PBS and frozen at -80°C. The total RNA was isolated from the pellet with a Total RNA Mini Plus kit (A&A Biotechnology, Poland).

Reverse transcription was performed with 5 μg of total RNA, 0.5 μg of Oligo(dT) reverse transcription starter, 4 μL of 5 × reaction buffer, 20 u of RiboLock RNase inhibitor, 2 μL of 10 mM dNTP mix (all reagents from Fermentas, Lithuania), and water to 20 μL for 60 min at 42°C. From each sample of cDNA, five real-time PCRs were performed for Gal, GalR1, GalR2, GalR3,

and GAPDH, each in triplicate. Composition of PCR mix was: 12.5 µL of FastStart Universal SYBR Green Mastermix (Roche, USA), 1 µL of cDNA preparation, and 1 µL of 5 mM primer mix (reverse and forward, Sigma, USA).

Sequences of primers were as follows: GAL: forward TGGGCCACATGCCATCGACA, reverse CGGCCTGGCTTCGTCTTCGG, GalR1: forward AGGATCACGGCGCACTGCCT, reverse GGGATT CCTTGCCAATGTGGCACT, GalR2: forward GCCAA GCGCAAGGTAACGCG, reverse GTAGGTGGCGC GGGTAAGCG, GalR3: forward GCACCACGCG CTCATCCTCT, reverse AGACCAGCGGGTTGAGG CAG. Porcine GAPDH was the housekeeping gene. The primers were: forward TTCCACCCACGGCAAGTT and reverse GGCCTTTCCATTGATGACAAG. Primers for GAL and GAPDH were designed using sequences of porcine GAL (NM_214234.1) and GAPDH (NM_001206359.1) sequences available in Gen Bank. Primers for GalR1 were based on porcine galanin receptor type 1 sequence (XM_003480426.2), GalR2 was based on predicted porcine galanin receptor type 2 (XM_003484313.1), and GalR3 was based on porcine predicted galanin receptor type 3 (XM_003355348.2). The primers were designed with Primer-BLAST software (http://ncbi.nlm.nih.gov). The reaction was performed in 7500 fast real-time PCR system (Applied Biosystems, USA) with the following thermal profile: initial denaturation 10 min at 95°C, denaturation 15 s at 95°C, and annealing 1 min at 60°C for 40 cycles. Data for galanin and its receptors' expression were normalised against GAPDH using software 7500 v. 2.0.2 (Applied Biosystems, USA). Data was statistically analysed with GraphPad Prism 5 software using one-way ANOVA and Tukey's post-test.

Results

A surprisingly high level of GAL expression was found in the mucosal lymphocytes in control animals. Following the experimental infection and development of enteritis, a very significant drop in the expression level of GAL was observed in the mucosal lymphocytes. Similar dramatic drop was also noted in the mucosa, where the expression of GAL was barely detected. However, a decrease in GAL expression was very small in the tunica muscularis, by approx. 1/8 (Fig. 1, Table 1).

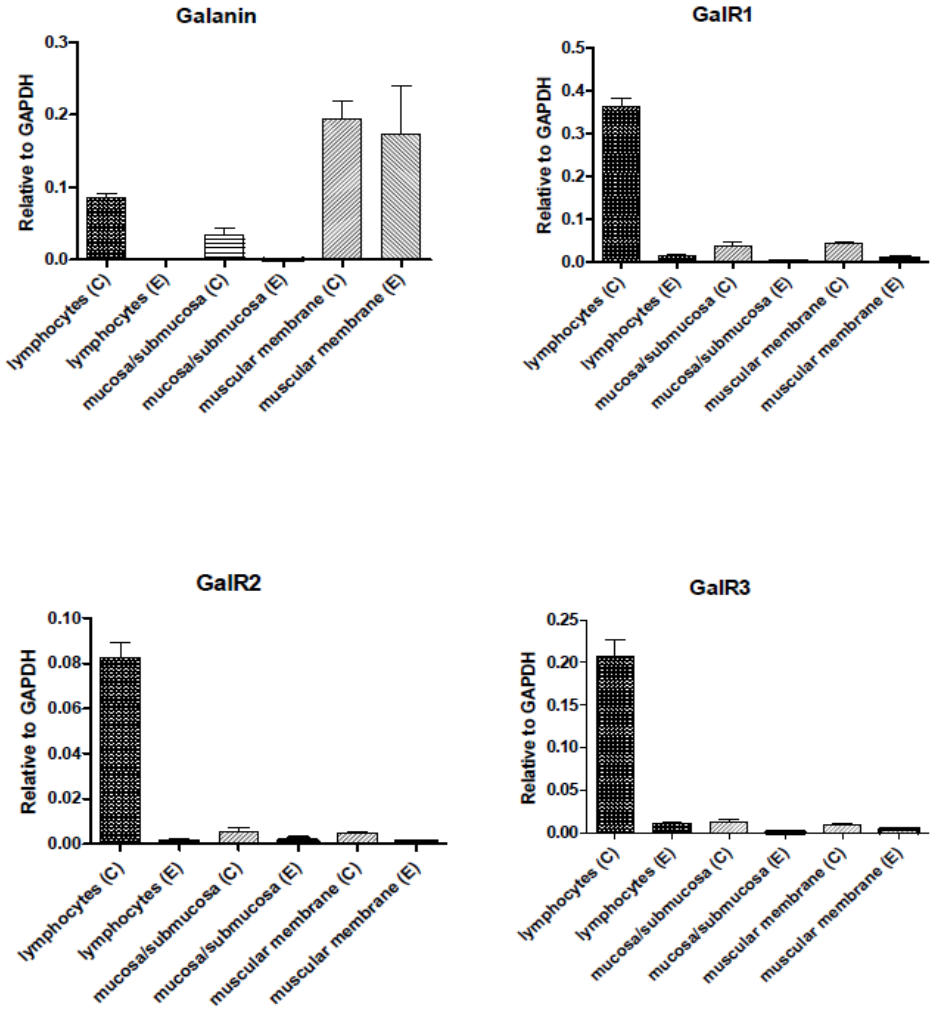


Fig. 1. Levels of expression of galanin, GalR1, GalR2, and GalR3 receptors in the colon of the control pigs (C) and pigs suffering from experimental dysentery (E). Expression levels shown relative to GAPDH expression level ± SEM

Table 1. Numerical values of expression levels of galanin, GalR1, GalR2, and GalR3 receptors compared to GAPDH expression (± SEM) in control (C) and experimental (E) pigs in three studied compartments of the colonic wall

	Lymphocytes		
	C	E	P value
Galanin	0.08520 ± 0.005434	0.001889 ± 0.0003469	***
GalR1	0.3634 ± 0.01935	0.01406 ± 0.003086	***
GalR2	0.08226 ± 0.007181	0.002055 ± 0.0004395	***
GalR3	0.2074 ± 0.01990	0.01098 ± 0.001673	***

	Mucosa/submucosa		
	C	E	P value
Galanin	0.03509 ± 0.008856	0.0004226 ± 0.00006193	**
GalR1	0.03829 ± 0.008232	0.001119 ± 0.0002181	***
GalR2	0.005454 ± 0.001833	0.001369 ± 0.001214	Ns
GalR3	0.01387 ± 0.002575	0.0009754 ± 0.0001394	***

	Tunica muscularis		
	C	E	P value
Galanin	0.1947 ± 0.02365	0.1739 ± 0.06652	Ns
GalR1	0.04293 ± 0.004704	0.009505 ± 0.001873	***
GalR2	0.005075 ± 0.0005626	0.001055 ± 0.0001651	***
GalR3	0.01039 ± 0.0009967	0.004380 ± 0.0007452	***

*** $P \leq 0.001$, ** $P \leq 0.005$, ns – not significant

In the case of GalR1 receptor, a significant decrease in its expression in mucosal lymphocytes in dysenteric animals was seen compared to high level of expression in control animals. Expression levels in the mucosa and tunica muscularis in control animals were much lower but a very significant decrease was also noticed in the dysentery-associated colitis (Fig. 1, Table 1).

Changes in the expression levels of GalR2 and GalR3 receptors were closely associated; however, the levels of the expression of these two receptors were different in control animals (Fig. 1, Table 1). In lymphocytes, GalR2 receptor expression level in control animals was 0.08, relatively to the level of GAPDH expression, while for GalR3 receptor, the expression level in control animals was much higher and amounted to ca. 0.2, in comparison with GAPDH expression. In the mucosa and tunica muscularis, the expression levels of these two receptors in control animals were approx. 10-fold lower. colitis caused a significant decrease in the expression of the two receptors in lymphocytes. In the mucosa and tunica muscularis, the drop was significant.

Discussion

Results of the study showed a significant decrease in the GAL expression in the colonic wall. However, the decrease was not uniform and affected mostly lymphocytes, mucosa and submucosa layers. In the tunica muscularis, the decrease of in the expression of GAL was moderate. The results suggest that the cells of the immunological system are significant site of GAL expression in the mucosa and submucosa. In part, due to a decreased GAL expression in them, the overall GAL expression level in this layer significantly decreased. It also seems that the GAL expression in muscular layer is largely unaffected by the inflammation. The obtained results are contradictory to those described previously in the dysenteric porcine colon (9), where the tissue concentration of GAL was increased approx. sevenfold in comparison to control animals. Previous morphological studies in dysenteric pigs also revealed that the number of GAL-positive neurons increased approx. twofold in submucosal and myenteric plexuses (18). However, it is difficult to estimate how GAL expressed by lymphocytes and neurons of the submucosal plexuses contributes to the total GAL content previously assayed in the dysenteric colon. It was found that the expression of GAL in isolated lymphocytes was very significant. However, the lymphocytes make only a fraction of the mucosa/submucosa tissue; hence the GAL expression in the mentioned layers was much lower. At the same time, the level of GAL expression in the tunica muscularis was very high and did not decrease significantly in inflammation. It is possible that the detected significant increase in GAL tissue concentration in the inflamed colon was due to the increased expression of the neuropeptide in the muscular layer (in the muscular nerve plexus). It must be also kept in mind that the

concentration of protein in the tissue is not directly dependent on the expression level of its mRNA, since it also depends on the storage, release, and decomposition rates of the protein.

However, our results concerning the changes of GAL expression in colitis presented here are in disagreement with our previous studies. It may be partially associated with difficulties to control the progress of the intestinal inflammation after *B. hyodysenteriae* inoculation. The only way to monitor the severity of enteritis is to observe the clinical condition and faeces. It is possible that during the course of the disease, the expression of different genes changes as it was found in enteritis in humans (24). There is a possibility that our previous studies were performed on animals in different stage of the disease (although the pathological lesions looked similar), or that the different properties of *B. hyodystenteriae* strain used for experimental infection evoked different course of the disease.

The distribution and role of GAL receptors in the organism is still under investigation. Only fragmentary data is available and we still cannot fully understand the role of the three known GAL receptors. Enteric smooth muscle cells express the GAL receptors, but some papers suggest that only GalR1 receptor is located on the colonic epithelial cells (13). It was found that in the dextran sulphate-induced colitis in mice, the expression of this receptor was upregulated, among others, in the colonic mucosal epithelium, where it is not normally expressed. This receptor may be responsible for the increased secretion of chloride ions into the colonic lumen as a mechanism leading to diarrhoea (13). In patients suffering from ulcerative colitis or Crohn's desease increased expression of GalR1 receptor was found (3). Cloning of GalR1 receptor revealed multiple binding sites for NF-κB transcription factor very strongly associated with inflammation (4). Other studies have shown that GalR1 receptor is also present on enteric neurons (1) and is responsible for regulation of enteric peristalsis (20). More detailed studies proved a wide distribution of GalR1 receptor in the intestinal wall (16). However, we found GalR1 receptor expression in the three studied colonic wall compartments and the expression of the receptor gene was decreased in these compartments in colitis.

GalR2 receptor appears to be involved in the functions of the nervous system. Its expression changes in the nerve cells in pathological processes, such as inflammation or axotomy (19). It seems that GalR2 receptor mediates GAL influence on peristalsis of the intestines (22). The expression of GalR2 receptor was found in all studied compartments of the colon, but its expression level was very low in comparison to GalR1 receptor. However, as in case of GalR1 receptor, its expression level decreased dramatically in the colitis.

The distribution and role of GalR3 receptor is not well known. It has restricted distribution in the central nervous system; however, its expression was found at a comparatively low level in many peripheral tissues of the rat (23). GalR3 receptor was found to be responsible for the regulation of microvasculature and oedema formation in dermatitis (17). A decrease in GalR3 receptor expression in mucosa/submucosa, isolated lymphocytes, and muscular layer was also demonstrated.

Our results showed a decrease in the expression of all three galanin receptors, although some reports indicated the elevation of their expression in inflammation (7, 14). The concern may be raised whether the decrease in the GAL receptors results from the deep destruction of the colonic wall leading to the disappearance of structures expressing the receptors. However, the histopathological examination revealed remarkable, but not extremely severe colonic wall destruction. The animals were alive, and the results of the GAL expression differed markedly from that for GAL receptors. In the previous paper we have also described the changes in the expression of three superoxide dismutases in the mucosa/submucosa, as well as in the tunica muscularis in pigs suffering from swine dysentery (the same individuals as described in the present paper), and found that no changes in the expression of SOD1 and SOD were detected in the mucosa/submucosa layer and expression of SOD3 raised significantly. In the muscular layer all SOD gene expression was raised significantly in dysenteric animals (5). The expression of some other genes, among them COX-2, which is involved in the development of inflammation (unpublished results), were studied, and an increase in COX-2 expression in colitis, as expected, was found. It is obvious that unexpected changes in the expression of GAL receptors in the colon of pigs suffering from swine dysentery are not due to the destruction of the colonic wall but reflect some phenomena occurring in the wall. However, the nature of these processes needs to be explained in further research.

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