Proliferative enteropathy (PPE)-induced changes in the expression of DBH, VAChT and NOS in the neurons of intramural ganglia of the porcine ileum

Z. Pidsudko, K. Wasowicz, J. Kaleczyc, M. Majewski, M. Lakomy

Faculty of Veterinary Medicine, University of Warmia and Mazury in Olsztyn, Poland

ABSTRACT: As enteric neurons are regarded to be highly adaptive in their response to various pathological states, including inflammation, it appears to be of interest to study the chemical coding of neurons in the intramural ganglia of the ileum wall in the course of porcine proliferative enteropathy (PPE) evoked by *Lawsonia intracellularis*. The study was performed on 12 juvenile pigs of the Large White Polish breed. The pigs were divided into the control (C, n = 6) group and the group consisting of pigs with clinically diagnosed *Lawsonia intracellularis* infection (E, n = 6). In E group animals the infection was confirmed with a PCR-based test. All the animals were sacrificed and segments of the ileum being pathologically changed were processed for double-labelling immunofluorescence using antibody against protein gene-product 9.5 (PGP 9.5) combined with antibody for dopamine β-hydroxylase (DβH), vesicular acetylcholine transporter (VAChT) or nitric oxide synthase (NOS). Immunohistochemistry revealed in the inner submucous plexus (ISP) and outer submucous plexus (OSP) an increase of the number of neurons containing DβH and VAChT in the E group. Interestingly, a decrease in the number of DβH- and VAChT-positive neurons in meynteric plexus (MP) ganglia of the E group animals was observed. The most remarkable difference in the chemical coding of enteric neurons between the control and PPE-suffering pigs was a significant increase of the number of NOS-positive nerve cells in the MP and OSP of the infected animals. The present results show that acetylcholine, catecholamines and NO may be involved in the regulation of functions of the porcine enteric nerve pathways not only under physiological, but also pathological conditions.

Keywords: ileum; enteric nervous system; biologically active substances; immunohistochemistry; porcine proliferative enteropathy; pig

However the gastrointestinal tract receives extrinsic autonomic and afferent innevation, the wall of the stomach and gut harbors an extremely important group of nerve cell bodies, sometimes called a "gut brain", which is responsible for the regulation of secretomotor activities of the alimentary tract at a local level. In the intestine of large mammals, such as pig, horse and dog two interconnected, but separate, intrinsic submucous plexuses can be discerned. One is located close to the muscularis mucosae, and is called an inner submucous plexus (ISP), the other one is located near the circular external muscle layer, and is called an outer submucous plexus (OSP) (Balemba et al., 1999, 2002a; Timmermans et al., 2001). Furthermore, the myenteric plexus (MP) situated between the longitudinal and circular muscle layers is involved in the regulation of enteric functions (Balemba et al., 1999; Lomax et al., 1999; Hens et al., 2000, 2002; Timmermans et al., 2001; Brehmer et al., 2002). Each of the above-mentioned plexuses contains a heterogeneous population of neurons with different functions and targets. Observation that a single enteric neuron may contain more than one transmitter or neuron-specific marker has led

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to the concept of chemical coding of enteric neurons related to their function and segmental position (Costa and Furness, 1984; Costa et al., 1984, 1992; Ekblad et al., 1984; Keast et al., 1984; Furness et al., 1987; Scheuermann et al., 1987; Barbiers et al., 1994; Timmermans et al., 1994, 2001). An important discovery was that neurons can change their chemical phenotype under pathological conditions (Csillik et al., 2003), including inflammation (Sharkey and Kroese, 2001; Ekblad and Bauer, 2004). These adaptive changes include both up- and down-regulation of neurotransmitter expression and induction of normally inactive genes.

Most of the data on the plasticity of the ENS have been collected in models of experimental intestinal inflammation in laboratory animals (Holzer, 1998; Evangelista, 2001; Feher et al., 2001; Sharkey and Kroese, 2001; Abad et al., 2003) and such studies performed on large farm animals are less numerous (Kaleczyc et al., 2004, 2007). On the other hand, the information on the adaptive changes of the enteric neurons in the course of natural inflammation associated with diseases affecting the gastrointestinal tract is very limited (Romanska et al., 1993; Balemba et al., 2001), and such data obtained in large animals are rare.

Pig is an important farm animal, in which gastrointestinal ailments cause economical losses (especially in young animals). On the other hand, due to the morphofunctional similarities between human and porcine alimentary tract this species is commonly used as a model animal of human diseases (Swindle et al., 1992). One of commonly occurring diseases of pigs associated with gastrointestinal disorder is proliferative enteropathy, a disease of pigs caused by the bacterium *Lawsonia intracellularis* (Lawson et al., 1993; Lawson and Gebhart, 2000). Contrary to some other inflammatory diseases of alimentary tract in which regressive (and even necrotic) changes occur (i.e. swine dysentery) the *L. intracellularis* infection induces in intestines proliferative changes affecting all components of the tissue. The proliferative changes may occur at different location in the intestinal tract, and also may be modified by secondary changes to primary lesions that alter the gross pathological appearance. This disease in pigs is associated with many specific lesions occurring in the lower ileum, and less commonly, in the large intestine. Usually, affected areas of intestine may show irregular patchy sub-serosal oedema, with small flecks of necrotic material on the surface of the thickened mucosa. In mild cases the thickening of the mucosa takes the form of small raised opaque islands within the normal epithelium. With increasing severity, the lesions become confluent and show an irregular nodular or folded surface. In our previous paper (for details see Pidsudko et al., 2008) we investigated changes in expression of a number of neuropeptides present in neurons located into the wall of intestine in the course of proliferative enteropathy. However, there is very little information concerning the distribution of neurons containing markers of small neurotransmitting molecules, like vesicular acetylcholine transporter (VAChT, a marker of acetylcholine), dopamine β-hydroxylase (DβH, a marker of catecholamines) or nitric oxide synthase (NOS, a marker of nitric oxide – NO) in the wall of the gut. Cholinergic transmission is involved in several key functions of the gut (Furness and Costa, 1974) and may be altered under a number of disease conditions (Burleigh, 1988; Bassotti et al., 1992). Noradrenaline (NA) is well established as the transmitter of postganglionic sympathetic neurons supplying the gastrointestinal (GI) tract (Furness and Costa, 1974) and adrenergic pathways are involved in the control of several key functions of the gut: they modulate vascular tone, inhibit motility and fluid secretion (Furness and Costa, 1974). NO plays an important role in mediating accommodation in the small bowel (Waterman et al., 1994) and colon (Ciccocioppo et al., 1994) as the inhibition of nitrergic pathways in the intestine facilitates distension-induced excitation and thus favors propulsion of intraluminal contents. NO synthase inhibitors were found to reduce the threshold volume required to trigger peristalsis in the small bowel (Waterman and Costa, 1994), and to reduce the latency for the initiation of the peristaltic reflex in the colon (Ciccocioppo et al., 1994). NO also is important factor in the pathophysiology of inflammatory bowel diseases (IBD). Its production is enhanced in IBD an increased NOS activity is observed in both clinical and experimental intestinal inflammation (Boughton-Smith et al., 1993; Miller et al., 1995; Miller and Sandoval, 1999).

This is why we decided to do additional studies on the influence of *L. intracellularis* infection on the changes in expression of VAChT, DβH and NOS in the intramural neurons of the porcine ileum. This data may shed some light on the involvement of cholinergic, catacholaminergis and nitrergic neurons in the inflammatory process associated with porcine proliferative enteropathy.

MATERIAL AND METHODS

The study was performed on 12 juvenile pigs of the Large White Polish breed (10 kg of body weight, age of six weeks) obtained from a commercial fattening farm in Lomianki (Poland). All the animals were housed and treated in accordance with the rules approved by the local Ethical Commission (conforming to the "Principles of Laboratory Animal Care", NIH publication No. 86-23, revised 1985). The animals were divided into the control (C, n = 6) group consisting of normal, clinically healthy animals and group consisting of pigs with clinically diagnosed *Lawsonia intracellularis* infection (E, n = 6). The *Lawsonia intracellularis* infection was confirmed with a routine PCR-based test at a State Veterinary Research Institute in Pulawy (Poland). All the pigs were sacrificed with an overdose of sodium thiobarbiturate (Thiopental, Sandoz, Austria; 40 mg/kg b.w., *i.v.*) and perfused transcardially with 4% buffered paraformaldehyde (pH 7.4). The ileums were cut out, their samples displaying most pronounced typical pathological changes were postfixed by immersion in the same fixative for several hours and finally they were stored in 18% sucrose until sectioning. Ten µm-thick cryostat sections of the tissue samples were processed for double immunofluorescence (Pidsudko et al., 2001) to study the distribution of the intramural nerve structures (visualized with antibodies against protein gene-product 9.5; PGP 9.5) and their chemical coding using antibodies against VAChT, DβH and NOS, as well as the secondary antibodies (Table 1). Thus, each mixture of primary antibodies applied contained antibody against PGP 9.5 (to visualize the enteric nerve structures) and antibody against one of the remaining substances. The labeled sections were studied and photographed with a Zeiss Axiophot fluorescence microscope equipped with epi-illumination and an appropriate filter set for FITC and Texas Red, and with a laser confocal microscope Bio-Rad Microradiance MR2. To determine percentages of particular neuronal populations, at least 500 of PGP-9.5-positive neuronal profiles were investigated for the presence of one of the biologically active substances in a particular ganglionated ileum plexus (MP, ISP and OSP) in each animal. The sections stained for the same combination of the antigens assigned to quantitative investigations were separated by at least 100 µm to avoid double-counting of neuronal somata. Only somata profiles containing nuclei were counted.

Statistical analysis was carried out with Student's t-test (GraphPad Prism v.2.0, GraphPad Software Inc., San Diego, CA). All results are expressed as means ± S.E.M. The differences were considered as statistically significant at $P \leq 0.05$.

Standard controls, and the omission and replacement of all primary antisera by non-immune sera were applied to test antibody specificity.

RESULTS

The samples of intestine taken for immunohistochemistry presented the macroscopic changes typical for proliferative enteropathy: thickening of mucosa and focal subserosal oedema. Histologically, the branched and widened intestinal crypts with pathologically altered epithelium were seen in the ileum wall.

Table 1. Antisera used in the study

Antigen	Code	Dilution	Species	Supplier
Primary antibodies				
PGP-9.5	13C4	1 : 2 000	mouse	Biogenesis, UK
VAChT	H-V006	1 : 8 000	rabbit	Phoenix Pharmaceuticals, USA
DBH	DZ 1020	1 : 500	rabbit	Affiniti, UK
NOS	8648C	1 : 5 000	rabbit	Cappel, USA
Secondary reagents				
Biotinylated anti-rabbit IgG		1 : 400	goat	Dako, DK
FITC-conjug. anti-mouse IgG		1 : 400	goat	Jackson Immun. Lab., USA
Streptavidin-conjug. CY_3		1 : 4 000		Dianova, Hamburg, GER

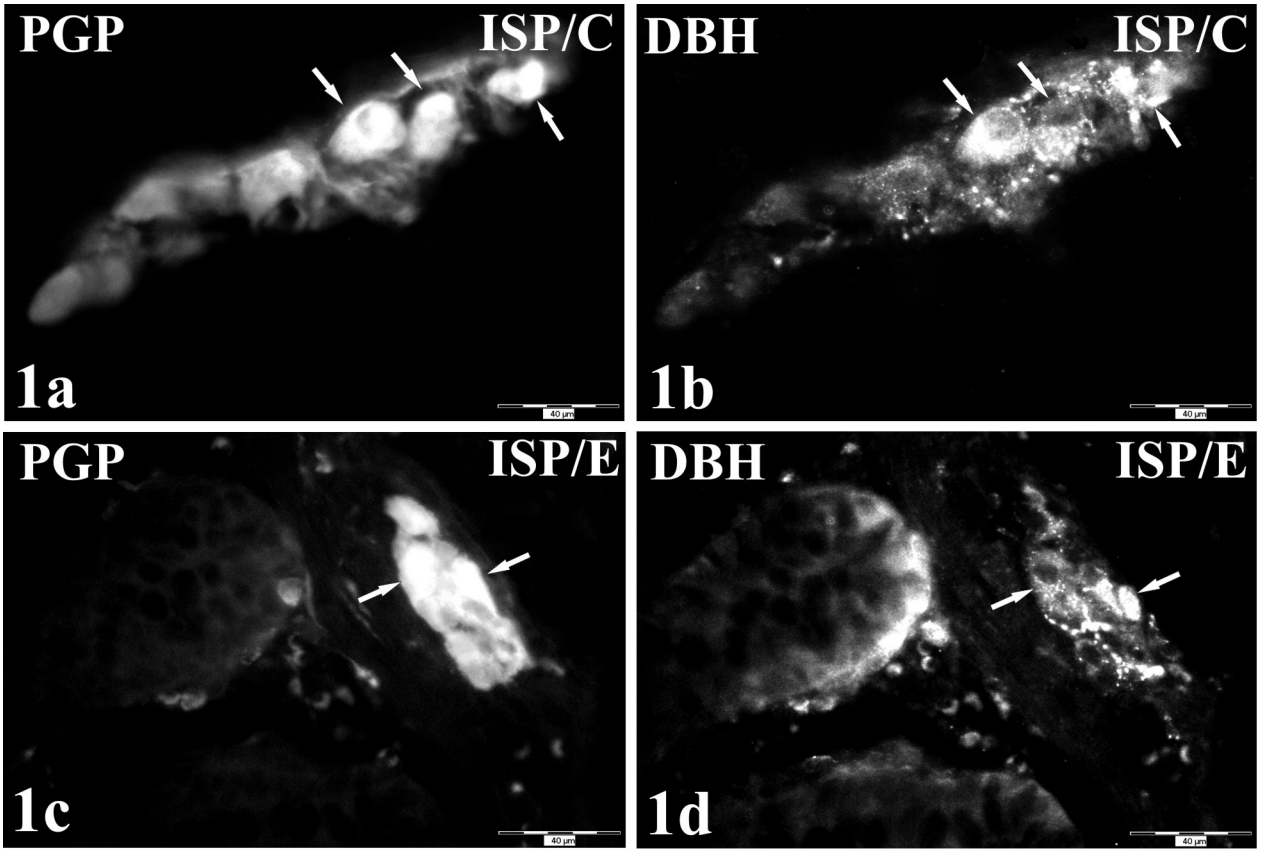

Figure 1a–b. The inner submucous plexus (ISP) of the ileum in the control (C) pig. The figures show the distribution of PGP 9.5- (**1a**) and DBH-positive (**1b**) nerve structures. Arrows in both figures point to neurons which are simultaneously positive to PGP 9.5 and DBH; bar 40 μm

Figure 1c–d. The inner submucous plexus (ISP) of the ileum in the pig with proliferative enteropathy (E). The figures show the distribution of PGP 9.5- (**1c**) and DBH-positive (**1d**) nerve structures. Arrows in both figures point to neurons which are simultaneously positive to PGP 9.5 and DBH; bar 40 μm

Table 2. Percentages of neuronal populations in the wall of the ileum in the normal (control) pigs and in the pigs with proliferative enteropathy (E)

			Myenteric plexus (± SEM)	Outer submucosal plexus (± SEM)	Inner submucosal plexus (± SEM)
DβH	Control pigs	NCB	35.75 ± 0.74	23.31 ± 0.71	46.36 ± 0.54
	E pigs	NCB	8.40 ± 0.57	33.48 ± 0.81	58.15 ± 0.77
VACHT	Control pigs	NCB	44.81 ± 0.74	37.94 ± 0.73	50.72 ± 1.04
	E pigs	NCB	13.88 ± 0.72	47.84 ± 0.74	59.22 ± 0.61
NOS	Control pigs	NCB	4.86 ± 0.68	12.53 ± 0.79	26.53 ± 0.83
	E pigs	NCB	40.79 ± 0.77*	35.57 ± 0.80*	37.75 ± 0.73

*$P \leq 0.05$

Immunostainings against PGP-9.5 revealed three distinct, separate, well developed ganglionated plexuses in the wall of the porcine ileum (Figures 1–3). They included two submucosal plexuses: inner submucosal plexus (ISP; Figure 1) and outer submucosal plexus (OSP; Figure 2), found between the muscularis mucosa and lamina propria, and in the submucosa, respectively, and myenteric plexus (MP)

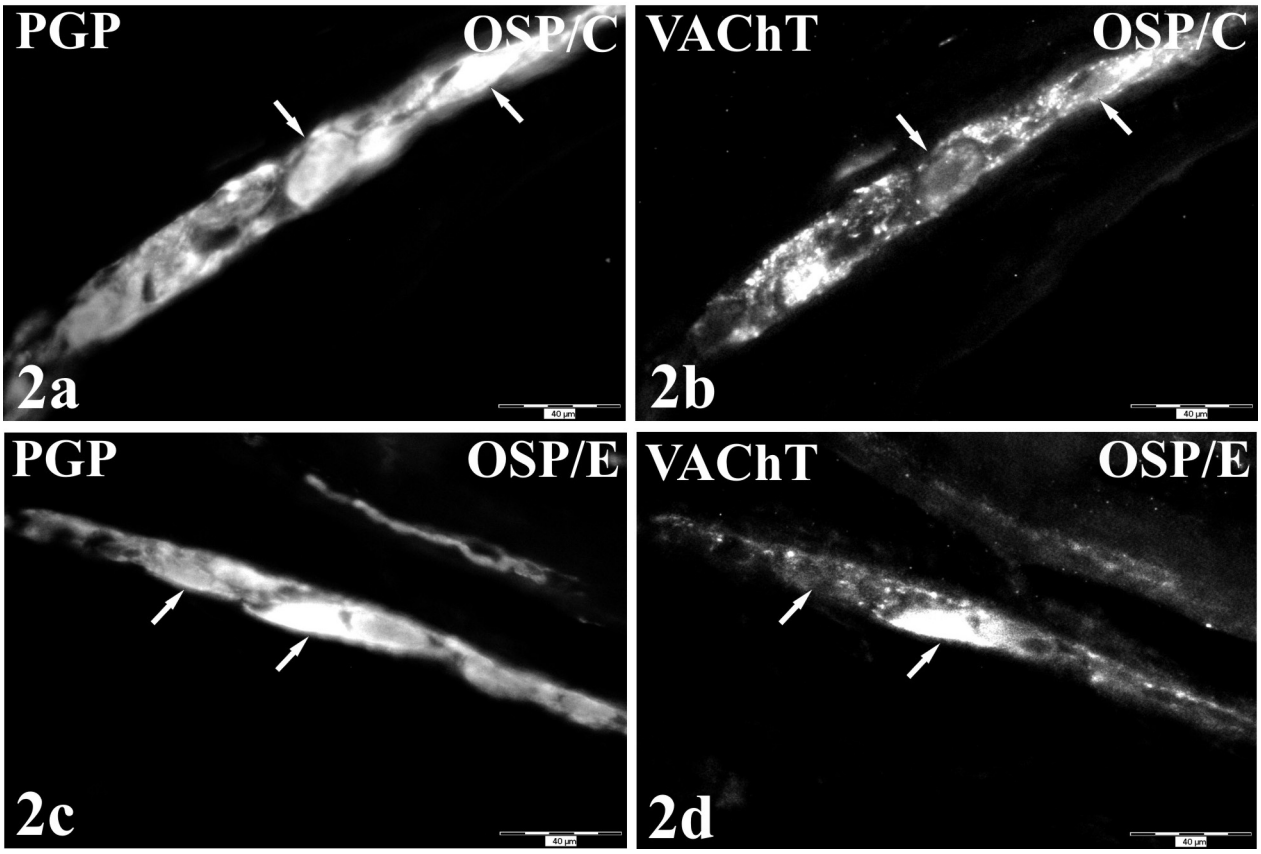

Figure 2a–b. The outer submucous plexus (OSP) of the ileum in the control (C) pig. The figures show the distribution of PGP 9.5- (**2a**) and VAChT-positive (**2b**) nerve structures. Arrows in both figures point to neurons which are simultaneously positive to PGP 9.5 and VAChT; bar 40 μm

Figure 2c–d. The outer submucous plexus (OSP) of the ileum in the pig with proliferative enteropathy (E). The microscope images show the distribution of PGP 9.5- (**2c**) and VAChT-positive (**2d**) nerve structures. Arrows point to PGP 9.5-positive neurons which are also VAChT-positive; bar 40 μm

Table 3. Semiquantitative evaluation of frequency of nerve fibre populations in the wall of the ileum in the normal (control) pigs and in the pigs with proliferative enteropathy (E)

			Muscle coat	Myenteric plexus	Outer submucosal plexus	Inner submucosal plexus	Mucosa
DβH	Control pigs	NF	+++	++	+	+	+
	E pigs	NF	+++	++	+	+	++
VACHT	Control pigs	NF	+++	++	+	+	++
	E pigs	NF	+++	++	+	+	++
NOS	Control pigs	NF	++	++	+	+	+
	E pigs	NF	++	++	+	+	+

+++ = numerous nerve fibres; ++ = moderate number of nerve fibres; + = small number of nerve fibres

located between the longitudinal and circular muscle layers of the ileum muscle coat (Figure 3).

In the ISP and OSP, immunohistochemistry revealed an increase in the number of neurons containing DβH (Figure 1d) and VAChT (Figure 2d) in the E group, as compared to control group (Figure 1b and 2b, respectively). Detailed numerical data are presented in Table 2. Interestingly,

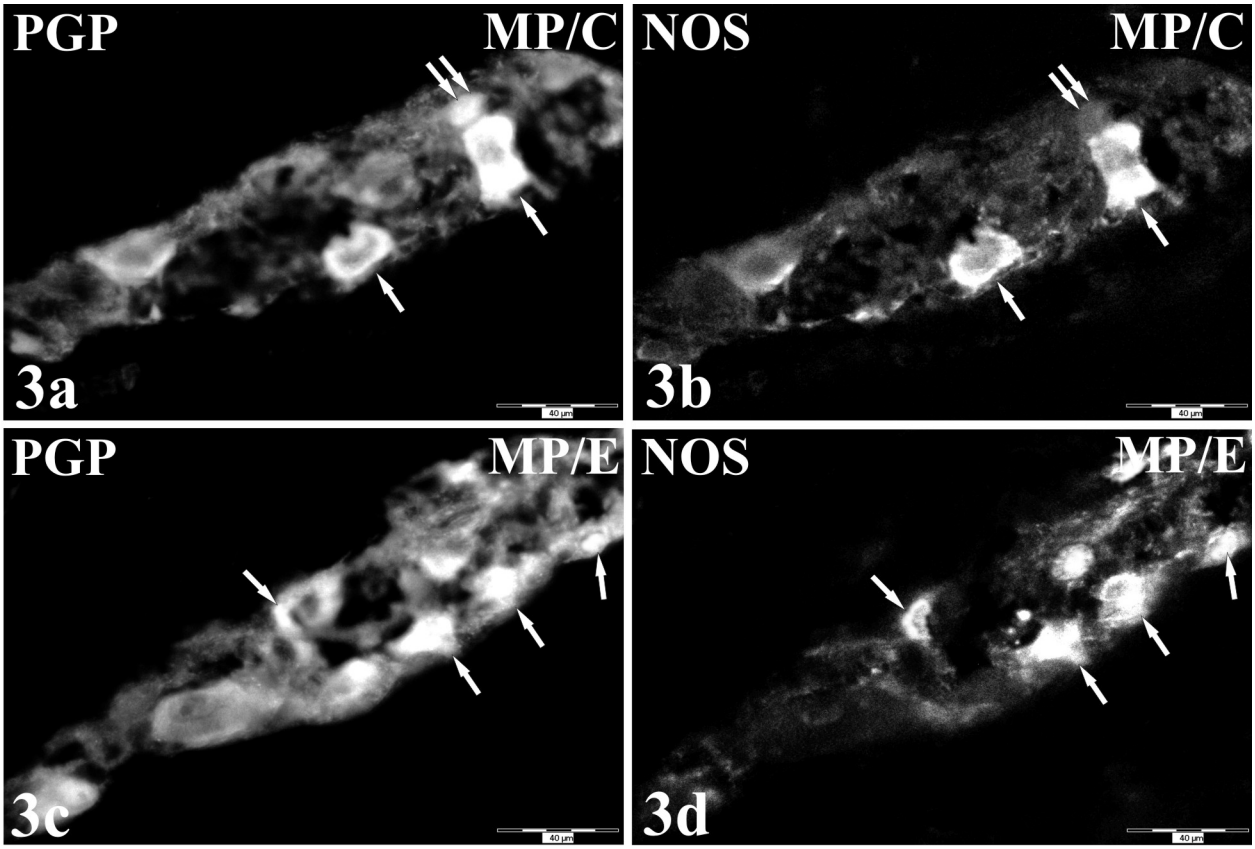

Figure 3a–b. The myenteric plexus (MP) of the ileum in the control (C) pig. The figures show the distribution of PGP 9.5- (**3a**) and NOS-positive (**3b**)nerve structures. Arrows point to PGP 9.5 neurons which are also NOS-positive. Double arrow points to PGP 9.5 neuron which is NOS-negative; bar 40 μm

Figure 3c–d. The myenteric plexus (MP) of the ileum in the pig with proliferative enteropathy (E). The figures show the distribution of PGP 9.5- (**3c**) and NOS-positive (**3d**) nerve structures. Arrows point to PGP 9.5-positive neurons which are also NOS-positive; bar 40 μm

a decrease in the number of DβH- and VAChT-positive neurons in MP ganglia of the E animals was observed. The most remarkable difference in the chemical coding of enteric neurons between the control and proliferative enteropathy-suffering pigs was a statistically significant increase in the number of NOS-positive nerve cells in the MP (Figure 3d) and OSP of the infected animals in comparison to control animals (Figure 3b).

Nerve fibres immunoreactive to DβH, VAChT and NOS were found in all layers of the ileum wall, i.e. in the mucosa, muscle coat as well as in the myenteric and submucosal plexuses (detailed **semi**-quantitative evaluation is presented in Table 3). In general, those observed in the muscle coat and myenteric plexus outnumbered those found in the remaining areas.

DISCUSSION

The present results revealed changes in the immunohistochemical characteristics regarding the expression of DβH, VAChT and NOS in the neurons located in the intramural ganglia of the ileum during PPE. In the pigs suffering from PPE the increase in the number of VAChT- and DβH-positive neurons was observed in OSP and ISP, while in MP the decrease in the number of these neurons was seen (although the differences were statistically not significant). The most remarkable difference in the chemical coding of enteric neurons between control and proliferative enteropathy-sick pigs was a highly increased number of NOS-positive nerve cells in the MP and OSP of the infected animals.

The results show clearly that the proliferative enteropathy affects the cholinergic, adrenergic and nitrergic subsystems of the enteric neuronal circuitry of the ileum. However, it is unclear whether these changes arise from the damage to enteric neurons evoked by the pathological process and, in such a case, alterations in chemical coding reflect the defence mechanisms induced in studied neurons, or the altered expression of DβH, VAChT and NOS is associated with active involvement of enteric neurons in fighting the inflammatory process.

The literature in the field contains many papers dealing with the chemical coding of intramural neurons in mammalian gastrointestinal tract. It is well known that in the gastrointestinal tract, acetylcholine is regarded as a major excitatory neurotransmitter and the prime regulator of the gastrointestinal motility. The release of acetylcholine from cholinergic nerve terminals is under well-regulated presynaptic control, involving specific neuronal receptors. Among them are purinergic P1 and P2 receptors, which, upon activation, enhance or inhibit the release of acetylcholine, depending upon the receptor subtype involved (Moody and Burnstock, 1982; De Man et al., 2003). Interestingly, there is some evidence that adenosine and ATP, which are natural ligands of P1 and P2 receptors, respectively, are generated at sites of inflammation (for a review see Cronstein, 1994). It is hypothesized that the immune response during chronic inflammation of the gut may directly affect the normal function of the enteric nervous system, but this has not been fully investigated.

Noradrenaline is well established as the transmitter of postganglionic sympathetic neurons supplying the gastrointestinal tract. Apart from the guinea pig intramural plexuses located in the proximal colon (which contains a substantial number of adrenergic cell bodies), all adrenergic fibers in the gut in laboratory animals are of extrinsic origin, as indicated by the disappearance of noradrenergic terminals after extrinsic denervation (for review see Furness and Costa, 1974)). In the guinea-pig noradrenergic axons in the gut are most numerous in the myenteric and submucous plexuses and around arterioles, but there is also evidence of a sparse noradrenergic supply to the circular muscle layer and to the mucosa (Gabella, 1979). With few exceptions, stimulation of sympathetic supply to sphincteric muscle is excitatory (Furness and Costa, 1987) due to a direct effect of noradrenaline on smooth muscle α-adrenoreceptor. In our study we observed for the first time such numerous population of adrenergic neurons (containing DβH) in all plexuses. The results obtained suggest that the adrenergic component of the enteric nervous system is affected by *L. intracellularis* infection. However, it is not known whether these changes are associated with the role noradrenergic neurons may play in local neural circuits of the inflamed porcine ileum during PPE.

Some studies have investigated transmitter release from the nerve terminals in ENS, and/or its consequence on the electrophysiological properties of enteric neurons, in inflammatory states. Both acetylcholine and noradrenaline release are depressed in *Trichinella*-infected rats (Swain et al., 1991; Collins et al., 1992; Ruhl and Collins, 1997) and after acute inflammation (Jacobson et al., 1997). The depression observed in infected animals was mimicked in both cases by preincubation of tissue with interleukin-1β (IL-1β) suggesting that it may be inflammatory mediator of this effect (Collins et al., 1992). This is further supported by studies in which interleukin-1 receptor antagonist was given to infected and inflamed rats. In this case noradrenaline release was enhanced as compared to infected/inflamed controls (Collins et al., 1992; Jacobson et al., 1997).

Another neurotransmitter, which is thought to be an important factor in the pathophysiology of the inflammatory bowel disease (IBD), is nitric oxide (NO). NO is involved in neuronal communication, regulation of blood flow and pressure, smooth muscle activity and intestinal motility, modulation of immunity and inflammatory reactions, neural defense mechanism and regeneration of axons during injury (Grozdanovic et al., 1994; Belai et al., 1997; Sigge et al., 1998; Bredt, 1999; Balemba et al., 2002b). Nitric oxide is generated intracellularly by an enzyme called nitric oxide synthase (NOS). An increase in NOS activity is observed in both clinical and experimental intestinal inflammation (Boughton-Smith et al., 1993; Miller et al., 1995; Miller and Sandoval, 1999). Neuronal NOS is localized in descending inhibitory motor neurons and some descending interneurons in the intramural plexuses (Furness et al., 1994; Costa et al., 1996). In experimental colitis in rats it was observed that the distribution and expression of the neuronal isoform of NOS was largely unaffected by this transmural inflammation (Miampamba and Sharkey, 1999). However, it was interesting that the inducible isoform of NOS was found in the myen-

teric plexus in the inflamed animals. This has also been reported in the guinea pig ileum in a similar model (Miller et al., 1995). This may represent some form of adaptation to inflammation because the appearance of inducible NOS correlated in time with the recovery from inflammation. However, in our study we have shown clear up-regulation of the neuronal NOS expression in myenteric plexus. It is interesting whether this phenomenon is attributed to species-related differences, or to the specific pathogenesis of PPE.

The present results show that cholinergic, adrenergic and nitrergic circuitries may play potentially important role in regulation of porcine enteric nerve pathways also under pathological condition, when the nervous system is "stressed", challenged, or afflicted by disease (such as PPE). However, the exact physiological relevance of adaptive changes observed remains to be elucidated in detail.

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Corresponding Author:

Zenon Pidsudko, Medicine, University of Warmia and Mazury in Olsztyn, Faculty of Veterinary, Department of nctional Morphology, Oczapowskiego Street 13, PL-10-719 Olsztyn-Kortowo II, Poland
Tel. +48 89 523 37 33, fax +48 89 523 49 86, e-mail: zenekp@uwm.edu.pl