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Original article

Changes in expression of calbindin 28 kDa in the small intestine of red kidney bean (*Phaseolus vulgaris*) lectin-treated suckling piglets

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Abstract

Calbindin (CB) is a calcium binding protein playing a role in calcium uptake and anti-apoptotic cellular protection. The expression of CB was immunohistochemically studied in the small intestine of normal and red bean kidney lectin-treated suckling piglets. In the duodenum and jejunum (but not ileum) of lectin-treated animals overexpression of CB was noted in chromogranin A-immunoreactive (CgA-IR) neuroendocrine (NE) cells. In both control and experimental group a small population of CB-IR NE cells exhibited the presence of somatostatin (but not serotonin, histamine or CRF). After the lectin treatment, an increased (however not statistically significant) immunoreactivity to CB was found in a small subpopulation of neurons of outer submucous (but not inner submucous and myenteric) plexus. It is suggested that there is a functional interaction between lectin administration and CB-expression in the porcine small intestine. Future studies will be needed to clarify this processes.

Key words: calbindin, neuroendocrine cells, enteric nervous system, red kidney bean lectin, small intestine, pig

Introduction

Calbindin 28kDa (CB) belongs to the large EF-hand family of cytosolic proteins which, because of their high affinities for calcium, are called calcium-binding proteins (CaBP). Using molecular techniques structure of the mammalian (including human) cDNAs and genes coding for CB have been identified, analyzed and cloned (Lomri et al. 1989, Varghese et al. 1989, Parmentier 1990). Several studies have revealed the wide distribution of CB in mammalian tissues, predominantly in central and peripheral nervous system. The expression of CB gene was detected in discrete regions of the rat brain including cells of dentate gyrus, inferior olivary nucleus, the nuclei of the trapezoid body, habenular nuclei, entorhinal cortex,

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the mammillary nuclei as well as in forebrain, brainstem gray matter and the posterior horn of the spinal cord (Abe et al. 1992). Different amounts of CB-immunoreactive (CB-IR) neurons and nerve fibres were additionally noted in the mammalian sympathetic (Masliukov et al. 2012), parasympathetic (Kuramoto et al. 1990, Richardson et al. 2003), sensory (Kuramoto et al. 1990) and enteric ganglia (Furness et al. 1990, Houghton et al. 1992, Arciszewski et al. 2009). However, neurons are not the only one source of CaBP since the expression of CB was also determined in many types of non-neuronal cells like ependymal cells (Abe et al. 1992), renal collecting tubule cells and the cortical collecting duct cells (Pollock and Santiesteban 1995), osteoblastic cells (Bellido et al. 2000), enterochromafin-like (ECL) cells and other subsets of neuroendocrine cells (Pochet et al. 1989, Katsetos et al. 1994, Andersson et al. 1998, Timurkaan and Tarakci 2007). It is generally thought that CaBP act as "trigger" or "buffers". CB is believed to belong to the latter group and as a regulator of the intracellular Ca^{2+} level is involved in variety of pivotal processes (for review see Schmidt 2012).

Weaning is one of the most stressful and critical period in lifetime of every mammalian species (with a special emphasis on the pig). Due to biochemical complicity of the weaning process, diet change, remodeling of the gut structure (maturation) and physiology as well as because of the decreased immunity leading to viral infections in the post-weaned piglets anorexia and in consequence relatively high mortality is noted (Madec et al. 2000, Lallès 2008, Grau-Roma et al. 2012). High economic looses in pork production are the main reason why many bio-scientists are involved in searching for an effective way to improve the ratio of successfully weaned pigs. In last years, red bean kidney (*Phaseolus vulgaris*) lectin has been added to the narrow list of useful commercial plant extracts which given orally prior to the weaning hasten the gut maturation and evoke beneficial changes in the gut physiology (Linderoth et al. 2006, Valverde Piedra et al. 2006). Although some basic aspects of lectin activities, like changes in villi morphology (Linderoth et al. 2006) and intestinal absorption (Rådberg et al. 2001), increase in the number of mucosal immune CD19+ and CD3+ cells (Prykhod'ko et al. 2009) or shift in chemical content of enteric neurons (Zacharko-Siembida et al. 2013) have been recently revealed many others are still obscure. Therefore, in the present study we applied immunohistochemical methods to evaluate the changes in the expression patterns of CB-like immunoreactivity in porcine small intestine occurring after the lectin treatment. In the co-localization studies the presence of serotonin, histamine, corticotrophin-releasing factor (CRF), and somatostatin (SOM) in CB-immunoreactive (IR) neuroendocrine NE cells were investigated in normal and lectin-treated piglets.

Materials and Methods

Animals and tissue preparation. All experimental procedures and animal care received approval from the Local Ethics Committee (20/2012) and stayed in agree with Principles of Laboratory Animal Care, NIH publications No. 86-23, revised 1985. All efforts were made to minimize the number of animals used and their suffering. Ten crossbreed Polish Landrace × Pietrain piglets (weighing approx. 4 kg) of both sexes, born at term and housed in standard farming conditions were used in the study. Three-day-old piglets were divided into a control (n=5) and an experimental group (n=5). In the experimental piglets oral dose of 160 HU/kg b.w. of a red kidney bean lectin preparation according to Pusztai and Watt (1974) was given. Control pigs were given 1 ml of saline to mimic the volume of the lectin administration. After 10 days the control and experimental piglets were sedated with intramuscular injection of azaperone (Stresnil, Janssen-Cilag GmbH, Germany; 0.5 mg/kg b.w.) and transported to the laboratory and killed by an overdose of sodium pentobarbital (Morbital, Biowet Pulawy, Poland; 50 mg/kg b.w.). Portions (approx. 10 cm) from all segments of the small intestine (duodenum, jejunum and ileum) were dissected out and rinsed in cold (4°C) 0.01 M phosphate-buffered saline (PBS; pH=7.3). Tissue samples were opened longitudinally, stretched, pinned onto a piece of balsa wood with serosal surface up and fixed by immersion for 48 h in cold (4°C) Stefanini's solution (containing paraformaldehyde and saturated picric acid). After the fixation the samples were placed for several days in cold 10% sucrose-containing cryoprotective Tyrode's solution (1 change per day). Finally the materials were mounted on a wooden block, embedded in O.C.T. compound and left to freeze in dry ice. Using a cryostat, serial longitudinal and transverse sections of 10 μm thickness were made. Every fifth section was placed on a glass slide (SuperFrost Plus, Menzel GmbH & CoKG, Germany) and stored at -20°C for further immunohistochemical studies.

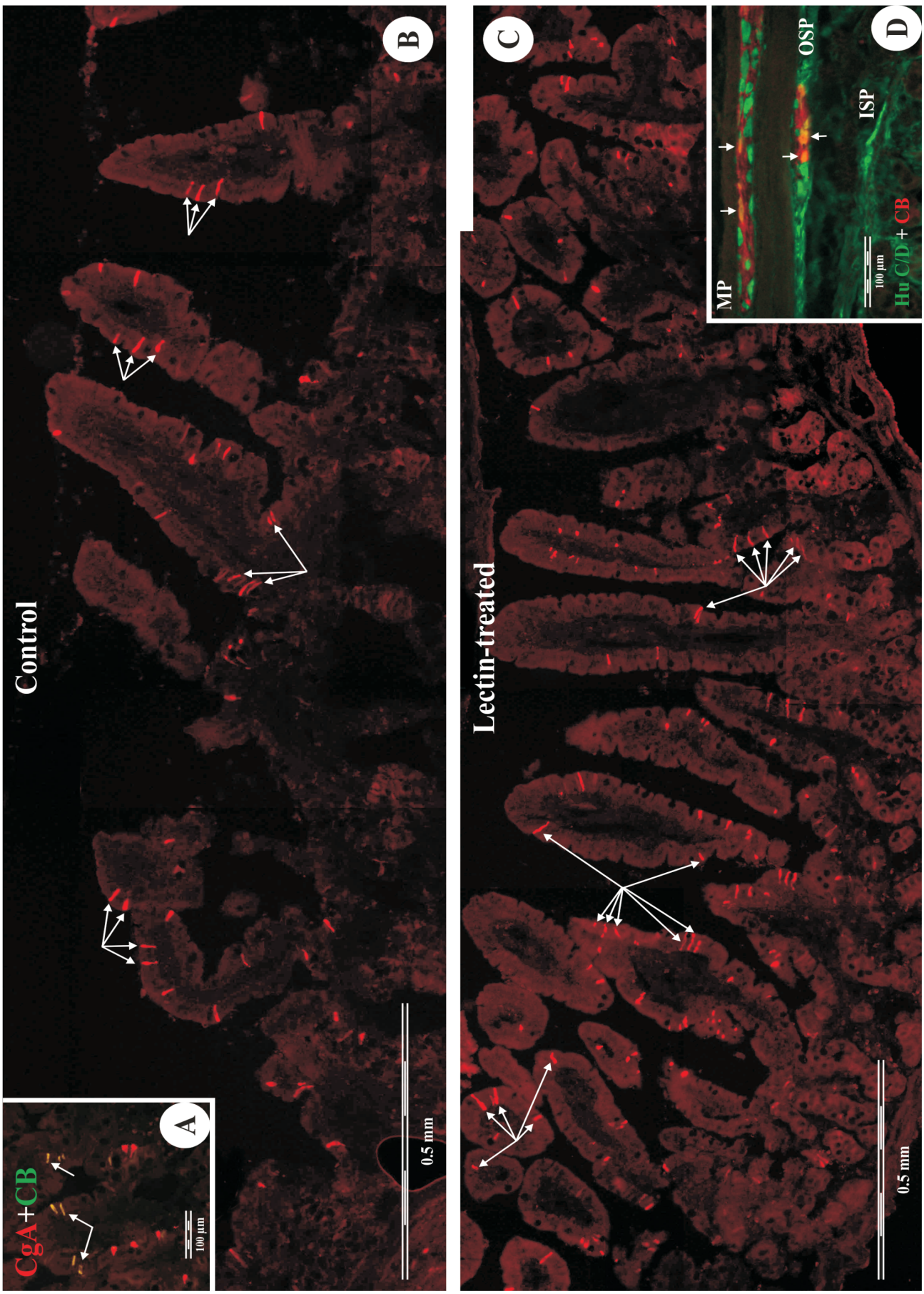
Immunohistochemistry. An indirect immunohistochemical procedure described elsewhere was applied (Arciszewski and Zacharko 2004). Briefly, the slides were dried out at room temperature (30 min, RT) and rehydrated in PBS. Next, the sections were immersed in a blocking solution of PBS containing 10% normal goat serum, 0.25% bovine serum albumin and 0.25% Triton X-100 (Sigma-Aldrich, MO, USA)

at RT. The sections were then incubated overnight in a humid chamber (RT) with a mixture of antibodies raised in two different species. In order to visualize calbindin 28 kDa (CB) in enteric neurons, mouse antibodies raised against general neuronal marker Hu C/D proteins (1:800; Molecular Probes, OR, USA; code A-21271) and rabbit anti-CB antibodies (1:2500; SWant, Switzerland; code CB 38) were applied. The presence of CB in NE cells was confirmed with the use of mouse anti-CB sera (1:2500; SWant, Switzerland; code 300) and rabbit antibodies raised against, widely expressed in subsets of NE cells, chromogranin A (CgA; 1:100, Abcam, UK; code ab45179). In the co-localization studies rabbit (or mouse if necessary) anti-CB antibodies were also mixed with mouse anti-serotonin (1:100; Abcam, UK, code 16007), rabbit anti-corticotrophin releasing factor (CRF; 1:80 Sigma-Aldrich, Germany, code C5348), rabbit anti-histamine (1:600; Pierce Thermo Scientific, USA; code PA118036) or rat anti-somatostatin (SOM; 1:300, Biogenesis, UK, code 8330-0009) antibodies. Next, after three further washes in PBS, the sections were then incubated (1 hour, RT) with a mixture of appropriate secondary antibodies. Texas Red-conjugated anti-rabbit goat IgG (1:400; MP Biomedicals, OH, USA), FITC-conjugated anti-rat goat IgG (1:400; MP Biomedicals) as well as FITC-conjugated anti-mouse goat IgG (dilution 1:400; MP Biomedicals) served as secondary antisera. The slides were finally rinsed in PBS (3x15 minutes) and covered with buffered glycerol (pH 7.4) supplemented with 0.1% para-phenylenediamine (Sigma-Aldrich, St. Louis, MO, USA) as an anti-fading factor. The observations were accomplished by using a spinning disk confocal microscope (BX-DSU Olympus, Nagano, Japan) equipped with appropriate interference filters for detection of Texas Red (545-580 nm; MWIY2) and FITC (470-490 nm; MNIBA2). Expression of two fluorescent levels was studied by interchanging filters. All images were captured using a digital color camera (DP-70, Olympus) and Cell^M software (Olympus). The specificity of primary antibodies was demonstrated by the preabsorption experiments, in which control sections were incubated with primary antibodies that had been inactivated with an excess of synthetic corresponding antigens (10-100 μg of blocking substance per 1 ml of diluted antiserum). The omission of primary antibodies, their substitution by normal, non-immune sera were also applied as a negative control. In all stained control sections no specific immunoreactions was observed.

Quantification and statistical analysis. In all portions of the small intestine of control and experimental animals, the numbers of CB-expressing NE cells were assessed by cell counting according to the following protocol. In each section stained with CB, lamina muscularis mucosae was first identified and measured. The integrity of lamina muscularis mucosae and mucosa was also checked. Then, in the sections with no damaged and interrupted mucosa (during sectioning) and lamina muscularis mucosae of the length not shorter than 10 mm, all CB-IR NE cells were identified and counted. In each segment of the small intestine, the mean from at least 5 slides from each animal (n=5 in control and n=5 in experimental group) was calculated. The numbers of CB-IR NE cells found in 1 cm of mucosa were expressed as a mean ± SEM. The visual semi-quantitative evaluation of the numbers of CB-IR NE cells was additionally done according to the following five-grade scale: absent, single, moderate, numerous and very numerous. The proportions of CB-IR NE cells co-expressing the biologically active substances (serotonin or SOM) were determined by cell counting and presented as a percentage relative to the total number of CB-IR NE cells. In each animal a random sample of no less than two hundreds of CB-IR NE cells was counted. The mean from at least 5 slides from each animal (n=5 in control and n=5 in experimental group) was calculated. The numbers of CB-IR enteric neurons were expressed as a percentage relative to the total number of Hu C/D expressing neurons. The obtained data were presented as mean ± SEM. The distribution patterns and densities of nerve fibres expressing CB were assessed visually according to the scale presented above. The one-way analysis of variance test (ANOVA) followed by Bonferroni's post hoc test were used to compare data between control and experimental groups. Differences were considered as significant at $P < 0.05$.

Results

General description. In the duodenum, jejunum and ileum from the control and experimental animals, the expression of CB was found in different numbers of NE cells. CB-IR NE cells were intensively stained, easily distinguishable from the background, and presented very characteristic morphology (Fig. 1A, 2C, 2D). The majority of CB-IR NE cells possessed axon-like extended cytoplasmic processes, however oval cells with no or very short and blunt extensions were also seen. In animals from the control and experimental groups, all CB-IR NE cells were immunopositive to CgA (Fig. 1A). In control and experimental animals, the presence of CB was also found in certain proportions of enteric neurons and nerve fibres of the small intestine (Fig. 1D).


A
CgA+CB
100 µm
B
Control
0.5 mm
C
Lectin-treated
0.5 mm
D
MP
OSP
ISP
100 µm
Hu C/D + CB

Expression of CB and co-localization of CB with serotonin, histamine, CRF, SOM in NE cells. In the duodenal mucosa of the control piglets, CB-IR NE cells were numerous (529 ± 44, n=5; Fig. 1B). When compared to the control, the number of CB-IR NE cells in the mucosa of lectin-treated animals (907 ± 85, n=5; Fig. 1C) was statistically higher ($P<0.05$). In both control and experimental piglets, the vast majority of CB-IR NE cells were found in the middle portion of the intestinal villi. CB-IR NE cells located in the duodenal crypts as well as in apical portion of the duodenal villi were moderately numerous. In the jejunal mucosa of the control piglets, CB-IR NE cells were predominantly seen in the middle portion of the villi (Fig. 2A). Lectin administration statistically increased ($P<0.05$) the average number of CB-IR NE cells present in 1 cm of the jejunal mucosa (to 263 ± 44 vs. 148 ± 32 found in control group; n=5; Fig. 2B) but had no influence on the distribution of CB-IR NE cells throughout villi. In sections from the jejunum of both control and experimental piglets, CB-IR NE cells were very rare in crypts and apical portion of villi. In control pigs, the mucosa of the ileum contained single CB-IR NE cells exclusively located in the middle portions of intestinal villi. After the lectin treatment the number of CB-IR NE cells present in the ileal mucosa was statistically unchanged when compared to the control (57 ± 19 vs. 42 ± 22, respectively). In the ileal mucosa of the lectin-treated animals no CB-IR NE cells located in crypts as well as apical portion of villi were found. In all segments of the small intestine of both experimental as well as control piglets, none of CB-IR NE cells exhibited the presence of serotonin (Fig. 2C) as well as CRF (Fig. 2D). Small subpopulations of CB-IR NE cells additionally co-storing SOM were found in the duodenum, jejunum and ileum of control animals (4.6 ± 1.1%, 3.9 ± 0.8% and 5.1 ± 0.9% respectively; n=5). When compared to the control, statistically similar ($P<0.05$) proportions of CB-IR/SOM-IR NE cells were noted in the duodenum (3.7 ± 1.0%; n=5), jejunum (4.4 ± 0.8%; n=5) and ileum (4.1 ± 1.2%; n=5) of the lectin-treated piglets (Fig. 2E). In control as well as lectin-treated piglets immunoreactivity to histamine has not been detected in NE cells, including those being CB-IR.

CB in enteric nervous system. In both control and experimental group, different proportions of CB-expressing neurons were detected in myenteric plexus (MP) as well as outer submucous plexus (OSP). In animals from both groups none of neurons located in inner submucous plexus (ISP) exhibited the presence of CB (Fig. 1D). In experimental and control piglets, moderate numbers of CB-IR nerve fibres were found to run between neurons of MP and OSP (but not ISP). In the duodenum, jejunum and ileum of the control animals as many as 14.6 ± 3.4%, 18.3 ± 3.2% and 19.4 ± 4.0% (respectively) Hu-IR/CB-IR myenteric neurons were found. No statistical changes were noted in percentages of CB-IR myenteric neurons in lectin-treated piglets (16.6 ± 3.2% in duodenum, 18.8 ± 3.7% in jejunum and 20.4 ± 2.9% in ileum). When compared to the control animals increased proportion of CB-IR OSP neurons were found in the duodenum (8.4 ± 3.4% vs. 4.9 ± 1.2% in control) and jejunum (8.0 ± 1.3% vs. 4.7 ± 0.6% in control), however these changes appeared to be statistically irrelevant. In both control and experimental animals the proportions of CB-IR OSP neurons found in the ileum were statistically similar (6.7 ± 1.1% and 6.9 ± 1.2% respectively). After the lectin treatment no visible changes in density and distribution patterns of CB-IR nerve fibres were noted.

Discussion

In the present study the abundant and region-specific expression of CB was observed in the small intestine of the normal piglets. IR to CB was limited to enteric neurons/nerve fibres and NE cells, however the numbers of CB-IR NE cells decreased gradually in aboral direction. The lectin stimulation substantially increased the numbers of CB-IR NE cells in the duodenum and jejunum (but not in the ileum). For several reasons CB-IR NE cells observed in the control and lectin-treated small intestine closely resembles ECL cells of the stomach. ECL cells are defined as NE cells of morphology similar to enterochromaffin (EC) cells, located exclusively in the oxintic part of the stomach, storing and secreting histamine (but not serotonin) and expressing CgA. Since virtually all ECL cells also co-store CB, several authors proposed to use this CaBP as a specific marker of the gastric ECL cells (Furness et al. 1989, Andersson

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Fig. 1. Expression of CB in the small intestine of the normal and lectin-treated piglets. (A) In double IHC stainings of the duodenum of the lectin-treated piglets the presence of CgA (red colour) in CB-IR neuroendocrine cells (green colour) is marked with arrows (CB-IR/CgA-IR cells are seen as yellow because of red and green colour fusion). The increased numbers of duodenal CB-IR NE cells (arrows) after the lectin treatment is illustrated in (B) and (C). In lectin-treated piglets the increased expression of CB was also found in certain subpopulation of duodenal and jejunal enteric neurons (D); CB-IR neurons are marked with arrows, MP – myenteric plexus, OSP – outer submucous plexus; ISP – inner submucous plexus.

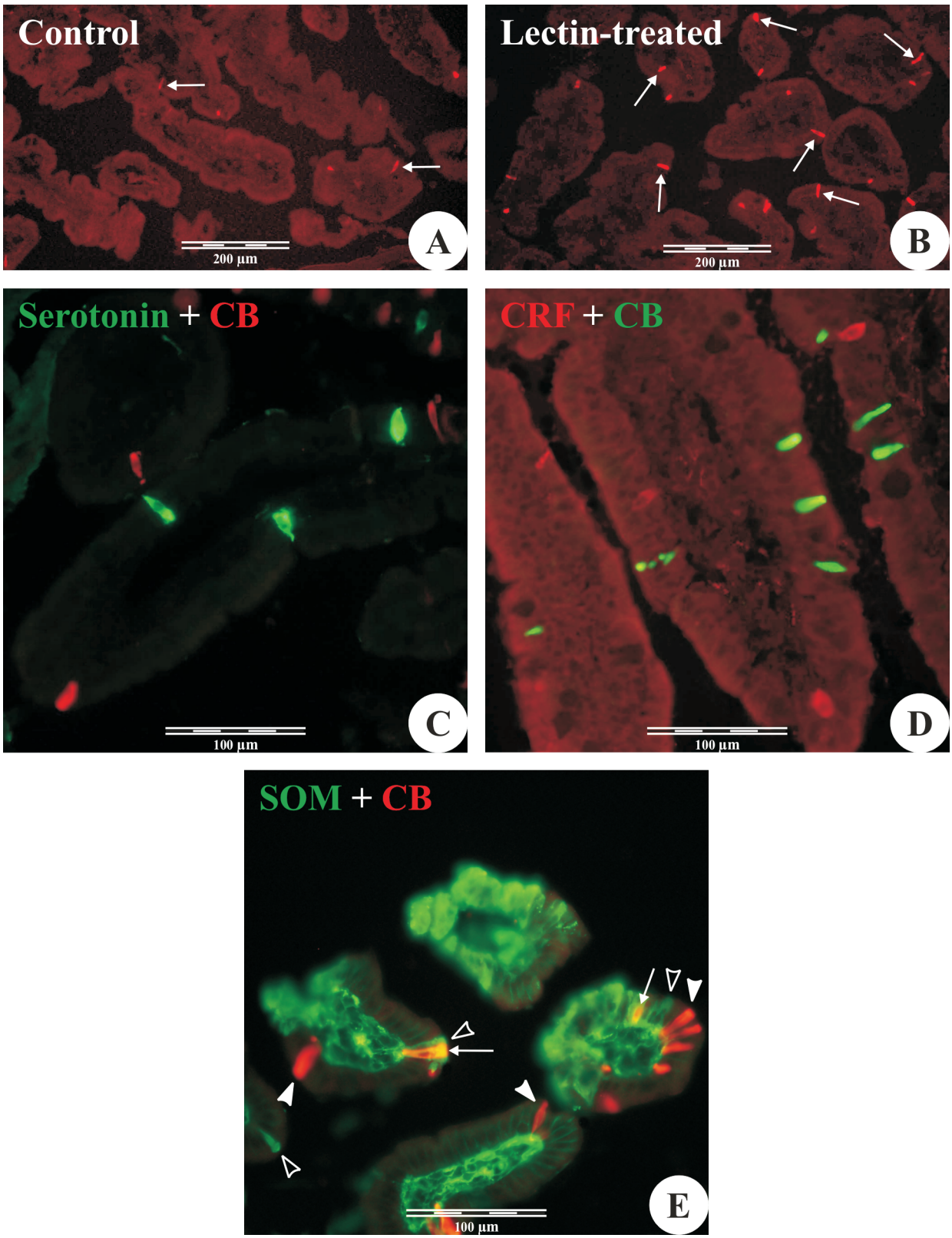

Fig. 2. CB-immunoreactivity in NE cells of the small intestine of the lectin-treated piglets. When compared to the control (A), in the jejunum of the lectin-treated piglets the increased proportion of CB-IR NE cells (arrows) was seen (B). Illustrations of the co-localization studies are presented in (C), (D) and (E). In the jejunum neither serotonin (C) nor CRF (D) was present in CB-IR NE cells. In (E) three different subpopulations of the duodenal NE cells are marked with: arrows (CB-IR/SOM-IR), arrowheads (CB-positive/SOM-negative) and empty arrowheads (CB-negative/SOM-positive).

et al. 1998). Taking into account the above it must be pointed out that, surprisingly, CB-IR NE cells identified in the present study fulfill some of criteria given for ECL cells (except location and histamine-expression). In the present study we found numerous CgA-IR/CB-IR NE cells with a shape similar to EC cells but since none of CB-IR NE cells expressed serotonin they definitely can not be considered as EC cells. Significance of this finding is unknown yet, however it may suggest the presence in the porcine small intestine a highly specialized subpopulation of NE cells. Further immunohistochemical analysis revealed that both in control and lectin-stimulated pigs nearly the same small subpopulation of CB-IR NE cells co-express SOM (but not CRF) which indicates that at least two different subpopulations of CB-IR NE cells can be distinguished. Noteworthy, in ECL cells of the rat, guinea-pig, dog and cat no CB/SOM co-localization was found (Buffa et al. 1989). In the small intestine, SOM secreted from D-cells is recognized as inhibitory neurohormone down-regulating the secretion of other hormones and playing a role in suppression of the gut contractility and blood flow circulation (see Van Op den Bosch et al. 2009 for review). In patients with inflammatory bowel disease the numbers of SOM-IR NE cells is substantially decreased (Watanabe et al. 1992). Therefore, it is possible that in CB-IR/SOM-IR NE cells SOM may inhibit CB release. In fetal sheep, the co-existence of CB and SOM, gastrin and glucagon (but not glucose dependent insulinotrophic peptide) in duodenal NE cells was observed (Houghton et al. 1992). Peripheral CRF secreted also from NE cells has been recognized as a modulator of intestinal disturbances occurring during the stress (Taché and Bonaz 2007) which may suggest that increased proportion of CB-positive/CRF-negative NE cells observed in the lectin-treated pigs is not stress-related.

It is widely known that one of the most important function of NE cells is recognition of luminal nutrients and appropriate secretory response in order to enhance food absorption but also to adapt to unfavorable conditions (Buchan 1999). Therefore, the numbers and chemical content of NE cells can be plastic during some pathological processes like inflammation or parasite infection (Lomax et al. 2006). In the present study we demonstrated that lectin stimulation also alters the number of CB-IR NE cells. Although the precise explanation of this finding has to be elucidated such situation from some points of view seems to be beneficial for the neonates development. Since in the small intestine CB was found to facilitate calcium transport across the mucosa (Wassermann and Taylor 1966) the higher calcium uptake would hypothetically explain a better skeleton calcification occurring after the lectin-treatment (Simmons et al. 1975, Izbicka et al. 1997, Kartsogiannis et al. 2008). During the weaning an increased apoptosis in epithelial cells is noted which frequently leads to disintegration of the intestinal barrier and viral infections (Zabielski et al. 2008). In this light, one of the key problem during weaning is to reduce apoptosis ratio. Apoptotic cell death-preventing properties of CB have been reported in relation to several cell types including PC12 cells (McMahon et al. 1998), osteocytes and osteoblasts (Liu et al. 2004), pancreatic β-cells (Rabinovitch et al. 2001) and neurons as well (Sun et al. 2011). Whether CB has a role in protecting epithelial cells from apoptotic cell death has not been reported yet, but higher expression of CB in NE cells after the lectin treatment may support this hypothesis. Neuroprotective properties of CB and its role against the excitotoxicity were widely reported in relation to many types of neurons (Rintoul et al. 2001, Yenari et al. 2001, Kim et al. 2010, Yuan et al. 2013). Noteworthy, in the present study we, after the lectin treatment, also found an increase (however not statistically significant) of CB content in certain subpopulation of submucous (but not myenteric) neurons what may suggest that lectin, at least to some degree, mediates CB-dependent promotion of survival of enteric neurons. In previous report, an increased levels of galanin and nitric oxide synthase (but not vasoactive intestinal polypeptide and substance P) were noted in the small intestine submucous neurons of the lectin-treated piglets (Zacharko-Siembida et al. 2013).

In conclusion, we herein described for the first time the changes in CB expression in the small intestine of the lectin-treated piglets. After the lectin stimulation increased numbers of CB-IR/CgA-IR cells were noted in the duodenum and jejunum (but not in the ileum). In co-localization studies the existence of SOM (but not serotonin and CRF) was revealed in a subpopulation of CB-IR NE cells. An increase of CB in a small supopulation of duodenal and jejunal OSP neurons was also determined. Our results may provide a new clue on the mechanisms underlying the weaning process, piglets welfare and maintenance. It is suggested that in the porcine small intestine, lectin stimulating CB-expression may be an effective and beneficial factor influencing various physiological aspects of GIT adaptation before weaning.

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