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Dietary Protein Level Influences on Neurotensin-immunoreactive Cells in the Chicken Ileum

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Neurotensin is secreted from intestinal N cells in response to the food ingestion. Influences of different dietary protein levels on neurotensin-immunoreactive cells in the chicken ileum were examined by using immunohistochemical and morphometrical techniques. The results showed that dietary protein had an obvious influence on neurotensin-immunoreactive cells in the chicken ileum. Four experimental groups were used, with dietary crude protein (CP) levels of 18% (control), 9%, 4.5% and 0%. Enteroendocrine cells showing neurotensin-immunoreactivity were located in crypts and villous epithelium in all groups. Most of the neurotensin-immunoreactive cells in the villous epithelium showed pyramidal or spindle-like shape with a long cytoplasmic process reaching the intestinal lumen, but cells with round or oval shape were found in the CP4.5% and 0% groups. Frequencies of occurrence of neurotensin-immunoreactive cells in the CP18%, 9%, 4.5% and 0% groups were 42.4±3.3, 36.6±2.2, 30.8±2.6 and 25.4±3.8 (cell count per mucosal area: cells/mm^2, mean±SD), respectively. There were significant differences in neurotensin-immunoreactive cell frequency between the control and lower CP level, 4.5% and 0%, groups. A significant correlation was found between frequency of occurrence of neurotensin-immunoreactive cells and daily protein intake. These results indicate that ingested protein is likely to be a potential signal for neurotensin production and secretion of N cells in the chicken ileum.

Key words: chicken, dietary protein, ileum, immunohistochemistry, neurotensin

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Introduction

The gastrointestinal tract is the main site involved in the process of digestion and absorption of ingested nutrients and also contains numerous endocrine cells which secret many kinds of peptide hormones including neurotensin. Neurotensin is a tridecapeptide separated from extracts of the bovine hypothalamus for the first time (Carraway and Leeman, 1973). The precursor protein of this hormone consists of 169−170 amino acids and is an exceptionally conserved polypeptide in rats, dogs, and cows (Dobner *et al*., 1987; Kislauskis *et al*., 1988). Neurotensin has been found in the central and peripheral nervous systems as well as in the gastrointestinal tract of various animal species (Polak and Bloom, 1982; Reinecke, 1985). It performs numerous physiological functions in the gastrointestinal tract, such as the regulation of gastric and intestinal motility (Thor and Rosell, 1986), the induction of pancreatic and biliary secretion (Gui *et al*., 2001), the enhancement of mucosal growth (Evers, 2006), and the increase of capillary permeability (Harper *et al*., 1984).

Enteroendocrine cells are designated with one or more letter and the neurotensin-secreting cell is known as the N cell. Helmstaeder *et al*. (1977) and Sundler *et al*. (1982) found that N cells in the mammalian intestine had an apical surface covered with microvilli and stored secretory granules in their basal cytoplasm. These morphological data indicate that intestinal N cells are “open-type” and may respond to intraluminal nutrients. Several studies carried out *in vitro* and *in vivo* have shown that nutrients such as amino acids, glucose and lipid stimulated neurotensin secretion from the intestine of mammalian species (Rosell and Rökaeus, 1979;

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Go and Demol, 1981; Holst Pedersen *et al*., 1988; Dumoulin *et al*., 1998; Drewe *et al*., 2008; Kuhre *et al*., 2015). Atoji *et al*. (1994) demonstrated that neurotensin-immunoreactive, open type cells were distributed throughout the whole gastrointestinal tract of several avian species. DeGolier *et al*. (2013) demonstrated that neurotensin was released from the chicken duodenum in response to intraluminal contents. Glucagon-like peptide (GLP) -1 is one of major peptide hormones secreted from the chicken intestine. Our previous studies in chickens demonstrated that GLP-1 secretion was influenced by ingested protein (Monir *et al*., 2014) and that GLP-1 colocalized with neurotensin in the same endocrine cells of ileum at a high ratio (Nishimura *et al*., 2017). These data suggest that neurotensin is also released from the chicken ileum in response to ingested protein; however, no study had yet tested this. In the present study, we aimed to clarify the influence of dietary crude protein (CP) levels on neurotensin-containing endocrine cells in the chicken ileum by immunohistochemical and morphometrical techniques.

Materials and Methods

Experimental Birds and Feeding

Twenty white leghorn chickens at 6 weeks of age were used in this study. They were divided into four groups, CP18% (control), CP9%, CP4.5% and CP0%, with 5 heads each and fed with the experimental diets containing different CP levels, 18%, 9%, 4.5% and 0%, for 7 days, after being fed with the control diet for 3 days. Details of dietary composition used and experimental feeding procedures in the present study are described in our previous article (Monir *et al*., 2014).

Tissue Samples

After completing the experimental protocols, chickens were sacrificed by decapitation under anesthetic condition. The distal ileum about 2 cm in length was immediately dissected out from each chicken as the tissue sample. Neurotensin is colocalized with GLP-1in the same cell at a high ratio in the distal ileum (Nishimura *et al*., 2017). After washing with 0.75% sodium chloride solution, tissue samples were fixed in Bouin's solution for 24 hr at room temperature and embedded in paraffin wax in the ordinary manner. Paraffin sections were cut at 5 μm with sliding microtome and subjected to immunohistochemistry for the detection of neurotensin.

Immunohistochemistry

The streptavidin-biotin method was used for the detection of neurotensin-immunoreactive cells (Guesdon *et al*., 1979). Paraffin sections treated with normal goat serum were incubated with rabbit polyclonal antibody against neurotensin (AB5496, Merck KGaA, Darmstadt, Germany) at room temperature for 24 hr. Biotin-labeled goat anti-rabbit IgG (AP132B, Millipore, CA, USA, diluted to 1:100) and streptavidin-polyHRP20 (SP20C, Stereospecific Detection Technologies, Baesweiler, Germany, diluted to 1:300) were used as the secondary antibody and the label for visualization of immunocomplex, respectively.

Morphometry

Morphometrical analysis was performed as described in our previous study (Hiramatsu *et al*., 2005). Cells showing neurotensin-immunoreactivity, and with clearly identifiable nuclei, were counted in each group and the area of the mucosal layer was measured. The cell count per area of the mucosal layer (cells/mm2) was then calculated to give the frequency of occurrence of neurotensin-immunoreactive cells. These measurements and quantifications were carried out using a computerized image analyzing system (KS400; Zeiss, Göttingen, Germany). Twenty areas were measured in each bird. One hundred areas in total were measured from the five chickens in each group

Statistical Analysis

Statistical analysis was conducted to determine the differences in the frequency of neurotensin-immunoreactive cells among the four groups using Tukey's method (Yanai, 2011). The GLM procedure (SAS/STAT) was carried out for multiple regression analysis between the daily protein intake and the frequency of occurrence of neurotensin-immunoreactive cells. The average value of the daily protein intake (g/day) of each chicken during the experimental period was calculated from the average value of the daily feed intake and CP level (%) of the experimental diet. Based on the national and the institutional regulations and guidelines, all animal experimental procedures were reviewed by the Committee for Animal Experiments of Shinshu University and finally approved by the president of the University.

Results

Many endocrine cells showing immunoreactivity for neurotensin antiserum were found in the ileal epithelium of all groups. Neurotensin-immunoreactive cells were mainly found in the epithelium of the intestinal villi and crypts in all the groups (Fig. 1a–d). There was no obvious difference in the distributional pattern of these cells among the four groups. Neurotensin-immunoreactive cells had flask- or spindle-like shape in the villous epithelium (Fig. 2a, c) and comma-like shape in the crypt (Fig. 2b, d), with apical cytoplasmic process reaching to the intestinal lumen. However, neurotensin-immunoreactive cells that were round or oval in shape were observed in the lower dietary CP level groups, especially in the CP0% group (Fig. 2c, arrow). The frequency of occurrence of neurotensin-immunoreactive cells in the CP18%, CP 9%, CP 4.5% and CP 0% groups were 42.4±3.3, 36.6±2.2, 30.8±2.6 and 25.4±3.8, respectively (Fig. 3, cell count per mucosal area: cells/mm^2, mean±SD). Significant differences were observed in the frequency of occurrence of neurotensin-immunoreactive cells between the control and lower CP level, CP 4.5% and 0%, groups ($p<0.05$). The values of frequencies were found to decrease as the level of dietary protein. There was a significant strong positive correlation between daily protein intake (X) and the frequency of occurrence of neurotensin-immunoreactive cells (Y) measured by using the GLM procedures for multiple regression analysis. The regression equation was: $Y=25.92+1.87\ X$ (Fig. 4).

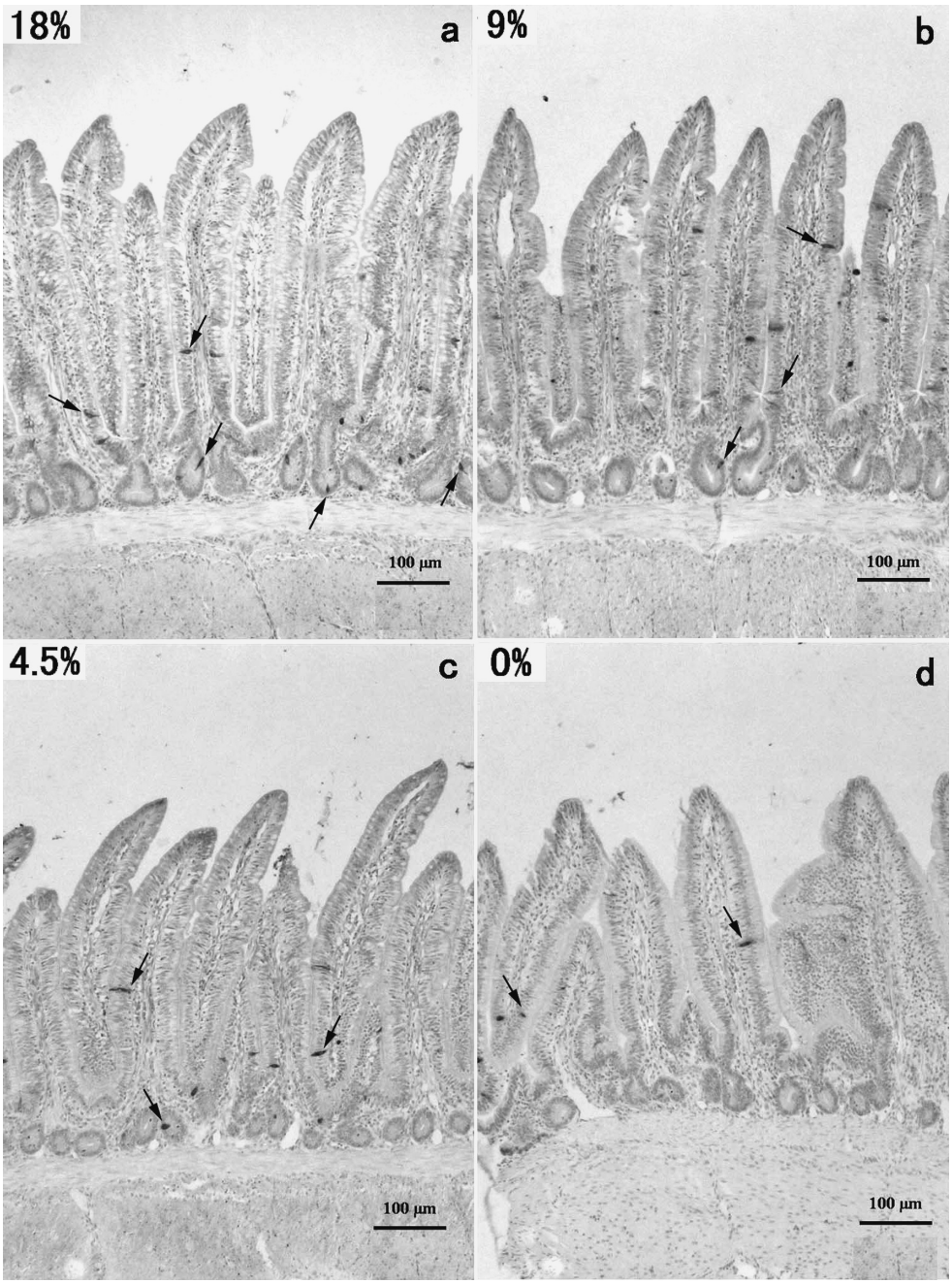

Fig. 1. **Low magnification images showing the distributional pattern of neurotensin-immunoreactive cells (arrows) in the ileum from chickens fed with four different dietary protein level, CP18% (a), 9% (b), 4.5% (c) and 0% (d).** Neurotensin-immunoreactive cells are distributed from crypt to higher part of villous epithelium in all groups, but their numbers are significantly decreased in CP4.5% and CP0% groups (c, d) compared with CP18% group (a).

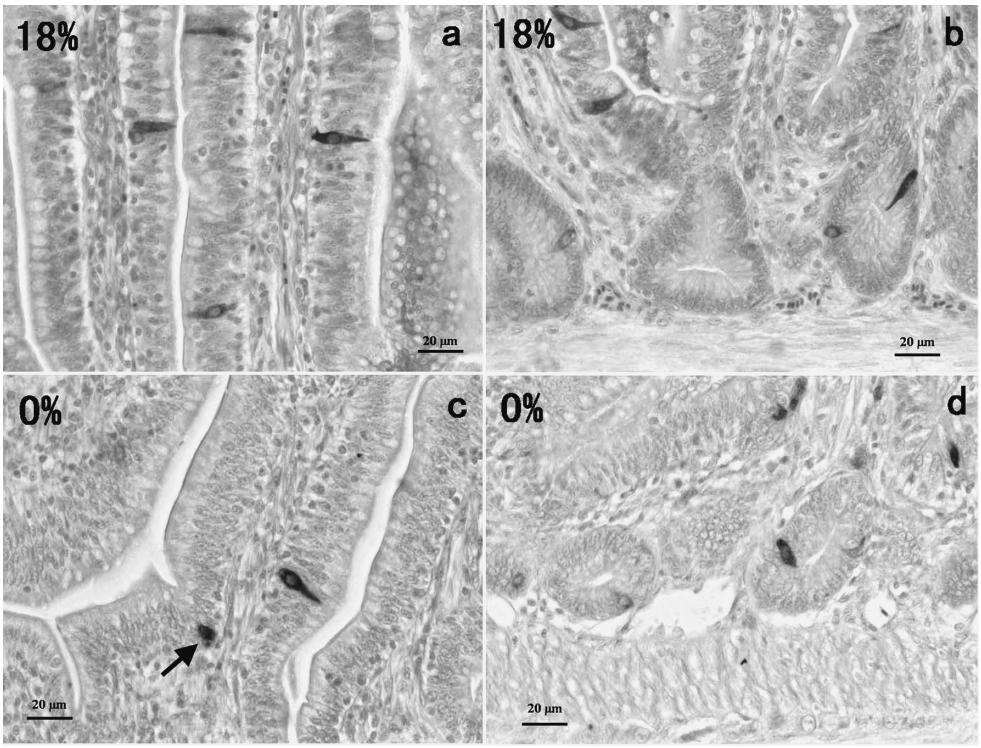

Fig. 2. **High magnification image of neurotensin-immunoreactive cells in villous epithelium (a, c) and crypts (b, d) of ileum from chickens of CP 18% (a, b) and CP0% (c, d) groups.** Neurotensin-immunoreactive cells display a flask or spindle-like shape with a long cytoplasmic process reaching to the intestinal lumen, but round or oval cell showing immunoreactivity for neurotensin is detected in villous epithelium of CP0% group (depicted by arrow).

Discussion

The present study demonstrated that dietary protein level influenced neurotensin secretion from the N cells of the chicken ileum.

Immunohistochemistry in this study showed that enteroendocrine cells exhibiting neurotensin immunoreactivity were scattered through the villous and crypt epithelium of the chicken ileum in the control and the three experimental groups. These neurotensin-immunoreactive cells have a long cytoplasmic process in contact with the intestinal lumen as mentioned by Atoji *et al*. (1994). This type of enteroendocrine cells is classified as "open-type" (Hiramatsu, 2019). Open-type enteroendocrine cells receive intraluminal nutrients as signals and release their hormones into the blood circulation (endocrine) or extracellular space nearby (paracrine) (Gribble and Reimann, 2016). Immunohistochemical and ultrastructural studies in mammals have indicated that open-type intestinal N cells have an apical surface covered with microvilli and contained secretory granules in their basal cytoplasm (Helmstaeder *et al*., 1977; Sundler *et al.,* 1982). Microvilli of open-type endocrine cells act as receptor sites for chemical signals, such as nutrients, in the intestinal lumen (Hashimoto *et al*., 1999; Breer *et al*., 2012). Sundler *et al*. (1982) reported neurotensin-immunoreactive cells having an apical process furnished with microvilli in the chicken antrum. Our previous study demonstrated similar ultrastructural features in chicken intestinal L cells which secret glucagon-like peptide (GLP) -1 (Nishimura *et al*., 2013) and that a large part of GLP-1-immunoreactive cells also exhibit immunoreactivity for neurotensin in the chicken ileum (Nishimura *et al*., 2017). It is possible that the neurotensin-immunoreactive cells observed in this study have an apical process covered with microvilli and receive nutrients as chemical signals.

Several *in vivo* and *in vitro* studies have reported stimulative effects of luminal nutrients, especially fats and fatty acids, on the secretion of neurotensin in mammals (Rosell and Rökaeus, 1979; Go and Demol, 1981; Holst Pedersen *et al*., 1988; Dumoulin *et al*., 1998; Drewe *et al*., 2008; Kuhre *et al*., 2015) and chicken (DeGolier *et al*., 2013). An *in vitro* study performed by Dumoulin *et al*. (1998) showed that an infusion of peptone, an enzymatic digest of protein, induced a marked increase of neurotensin in the rat ileum. However, it is not clear if ingested protein influences neurotensin secretion directly. Morphometrical analysis in this study

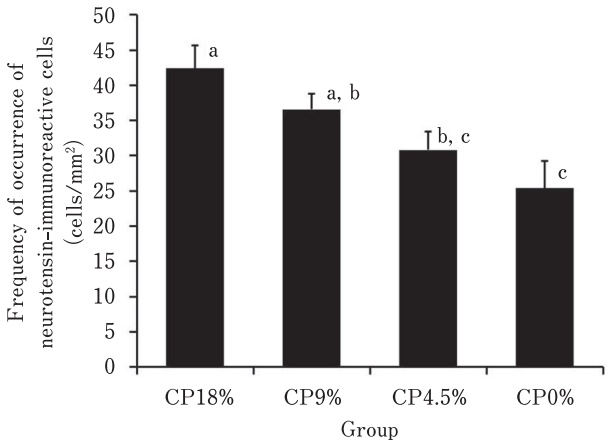

Fig. 3. **Frequencies of occurrence of neurotensin-immunoreactive cells in ileum from chickens fed with four different dietary protein levels (CP18%, 9%, 4.5% and 0%).** Values are represented by the average number of neurotensin-immunoreactive cells per 1 mm^2 mucosa. There are significant differences between different alphabets (a, b and c) ($p<0.05$, error bar: standard deviation).

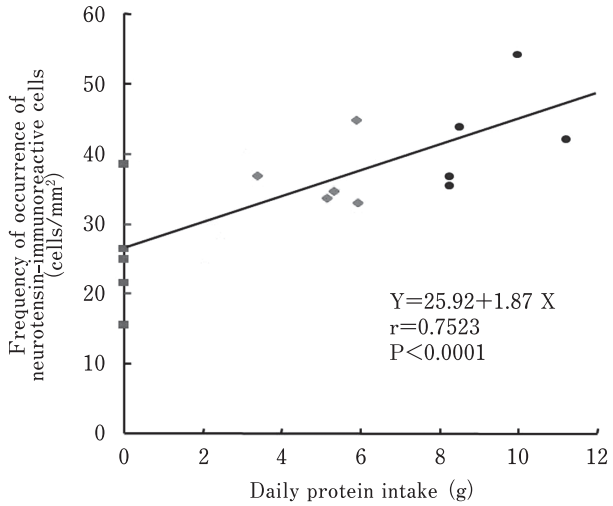

Fig. 4. **Regression line of frequency of occurrence of neurotensin-immunoreactive cells (Y) on the daily protein intake (X).** The equation of this line is Y=25.92+1.87X, $p<0.0001$.

revealed that there were significant differences in the frequency of occurrence of neurotensin-immunoreactive cells between the control and lower CP level (4.5% and 0%) groups. The frequency of neurotensin-immunoreactive cells was highest in the control group and decreased as dietary CP level was reduced. Moreover, multiple regression analysis showed a strongly positive correlation between daily protein intake and the frequency of occurrence of neurotensin-immunoreactive cells. El-Salhy *et al*. (2016) described that a change in diet not only affected the release of gastrointestinal hormones, but also altered the densities of the gut endocrine cells. In fact, the change in dietary protein level altered the frequency of occurrence of GLP-1-immunoreactive cells in the chicken ileum (Monir *et al*., 2014). In addition, neurotensin-immunoreactive cells that were round or oval in shape were found in lower CP level groups, especially in the CP 0% group. Endocrine cells in a round or oval shape were found in the ileal epithelium of the fasted chicken and degenerating features, i.e. vacuoles in the perikaryon and lobulated nuclei, were observed in these cells at the ultrastructural level (our unpublished data). This phenomenon suggests that neurotensin-immunoreactive cells are going to be degenerated in the CP0% group because of the absence of luminal proteins. Thus, the present morphometrical data indicate that dietary protein is one of the important factors which control the activity of N cells in the chicken small intestine.

Colocalization of plural hormones in the same enteroendocrine cell has been reported in mammalian species. Egerod *et al*. (2012) reported coexpression of neurotensin with some other hormones, such as cholecystokinin, secretin and GLP-1, in the mouse intestine. Svendsen *et al*. (2015) demonstrated coexpression of neurotensin with GLP-1 in the rat small intestine. Our previous study detected three types of enteroendocrine cells in the chicken ileum on the basis of the colocalizational pattern of GLP-1 with neurotensin (Nishimura *et al*., 2017). According to that study, cells containing both GLP-1 and neurotensin were more frequently observed than cells containing either hormone in the chicken ileum. Moreover, GLP-1-immunoreactive cells also decreased in density with reduced protein intake (Monir *et al*., 2014). Judging from these findings and the present data, it is possible that enteroendocrine cells which contain both neurotensin and GLP-1 are sensitive to dietary protein, but more systematic experiments are necessary to establish this for certain.

In the present experimental diets, cornstarch was included to maintain the constant energy density in the all groups (ME =2847−2850 kcal/kg DM). Several studies on mammals indicated that carbohydrate influenced the secretion of neurotensin from intestinal N cells in a dose-dependent manner (Dakka *et al*., 1993; Dumoulin *et al*., 1998; Kuhre *et al*., 2015). If there was any effect of the increased level of cornstarch on N cell frequency and morphological features in the chicken small intestine, it could have been observed in the lower dietary CP groups. In the present results, however, such effects were not observed. This therefore indicates that cornstarch does not have stimulative influences on N cells in the chicken small intestine.

In conclusion, dietary protein level functions as an effective stimulator for neurotensin secretion from N cells in the chicken ileum.

Conflicts of Interest

The authors declare no conflict of interest.

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