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Histological studies on the mechanisms for the regulation of glucagon-like peptides (GLPs) in the chicken small intestine

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Abstract

Histological studies on the mechanisms for the regulation of glucagon-like peptides (GLPs) in the chicken small intestine

Glucagon-like peptides (GLP-1 and GLP-2) are most important in the regulation of gastrointestinal growth and intestinal proliferation in chicken. These meal-induced peptides perform lots of important physiological actions and related with intestinal activities.GLP-1 is one of the incretin hormones and relates to the intestinal motility, and GLP-2 is an intestinotrophic peptide that stimulates intestinal growth. The mechanisms of GLPs secretion in chicken small intestine were investigated using the morphological techniques in this dissertation clarifying the following points. (1) GLP-1 and GLP-2 were colocalized in the same endocrine cells, L-cells, using double immunofluorescent technique. L-cells showing GLP-1 immunoreactivity only were observed in the epithelium of the middle part of intestinal villi. These results indicate that GLP-1 and GLP-2 are secreted from the same cells separately. (2) Restricted feeding influenced on the distribution and morphology of GLP-1 and GLP-2-immunoreactive cells in the chicken small intestine. The control and two experimental groups (50% and 25% feed supply) were set in this study. The frequency of occurrence of cells showing immunoreactivity for GLP-1 or GLP-2 was significantly increased in 25 % feed supply group. These results indicate that the quantity of feed intake is one of the signals stimulating the secretion of GLPs from chicken intestinal L-cells.

(3) Protein ingestion influenced on the frequency and morphology of GLP-1 and GLP-2-immunoreactive cells in the chicken small intestine. The control (CP 18%) and three experimental groups (CP 9%, CP 4.5% and CP 0% feeding groups) were set in this study. Low protein ingestion induced morphological alterations of GLP-1 and GLP-2-immunoreactive cells. Frequencies of GLP-1 and GLP-2-immunoreactive cells were significantly decreased in lower protein groups (CP 4.5% and CP 0%) compared with the control group. These results indicate that the protein ingestion is one of the strong stimuli to the GLPs secretion. In conclusion, it is revealed that GLPs secretion from intestinal L-cells is controlled by the quantity and quality of feed in chickens. Moreover, it is likely that GLP-1 and GLP-2 are secreted from the same cells separately. This dissertation discovered the important aspects of GLPs that has not previously been reported in chickens.

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Contents

Chapter I

General Introduction

General Introduction

The biological actions of the glucoregulatory hormone, glucagon from the pancreas, have been first identified over 90 years ago (Kimball and Murlin, 1923). The structure and biological importance of gut glucagon remained poorly understood for decades (Orci *et al*., 1968; Polak *et al*., 1971; Knudsen *et al*., 1975; Tanaka *et al*., 1979). Glucagon-like bioactivity in the gut was well-known for many years. After the isolation of the cDNAs and proglucagon (PG) encoding genes (Bell *et al*., 1983a, b; Lopez *et al*., 1983; Heinrich *et al*., 1984), two novel glucagon-like peptides (GLPs), GLP-1 and GLP-2, were found as potential bioactive peptide hormones. For a while it was thought that GLP-2 moiety was an evolutionary late addition to the gene, since bird proglucagon appeared to contain only a single glucagon-like peptide corresponding to GLP-1 (Lund *et al*., 1982). Subsequent research, however, established that the proglucagon genes in bird also contain the GLP-2 encoding sequence which is sequestered by differential splicing upon pancreatic expression of the gene (Irwin and Wong, 1995) but remains in sequence upon intestinal expression. GLPs are derived from a common precursor protein by alternative tissue-specific proteolytic cleavages in L-cells of the intestine, α-cells of the endocrine pancreas, and neurons in the brain. Non-mammalian vertebrates such as chicken, fish, frog, and lizard contain two proglucagon genes, and use alternative RNA splicing for the generation of proglucagon mRNA transcripts that encode for GLP-1 but not GLP-2 in the pancreas and both GLP-1 and GLP-2 in the intestine (Irwin and Wong, 1995; Chen and Drucker, 1997). The differences in the proglucagon products in these tissues are, therefore, due to tissue-specific, differential, posttranslational processing of proglucagon (Mojsov *et al*., 1986; Ørskov *et al*., 1986). The proglucagon gene is also expressed in certain neurons in the nucleus of the solitary tract in the brain stem (Larsen *et al*., 1997). The intestinal processing of proglucagon by prohormone convertase 3 (PC 3) results in the formation of GLP-1 (corresponding to PG 78-107 amide), GLP-2 (PG 126-158), intervening peptide-2 (IP-2,

PG 111-122 amide) and glicentin (PG 1-69) (Dhanvantari *et al*., 1996; Nian *et al*., 1999). Glicentin may be further cleaved to glicentin-related pancreatic peptide (GRPP, PG 1-30) and smaller amount of oxintomodulin (PG 33-69) (Holst, 1997; Kieffer and Habener, 1999). A large number of studies in rodents, larger mammals and humans have determined that both GLP-1 and GLP-2 are secreted in a nutrient-dependent manner (Elliott *et al*., 1993).

GLP-1, a 36-amino-acids peptide hormone, was first identified following the cloning of cDNAs and genes for proglucagon in the early 1980s. Synthesized in two principal major molecular forms, as GLP-1(7-36) amide and GLP-1(7-37), it regulates blood glucose and promotes glucose homeostasis by stimulating pancreatic insulin synthesis and secretion, and islet cell proliferation and neogenesis while inhibiting the secretion of glucagon (Drucker, 2001a). In addition, GLP-1 controls nutrient absorption through its inhibitory effects on gastric emptying and food intake regulation (Kieffer and Habener, 1999). Considering these properties, GLP-1 is under investigation as a potential incretin hormone regulating physiological as well as biological functions in animals and birds. The potential function of GLP-2, a 33-amino-acids peptide hormone, is thought to play a role in intestinal growth and nutrient absorption by maintaining the integrity of epithelial cells (Drucker, 2001b; Burrin *et al*., 2003). Secreted by enteroendocrine L-cells in response to the presence of intestinal nutrients, GLP-2 has been found to promote crypt cell proliferation and suppress apoptosis in mucosal epithelial cells (Burrin *et al*., 2003; Estall and Drucker, 2006). The actions of GLP-1 and GLP-2 are mediated via unique G protein-coupled receptors. The GLP-2 receptor (GLP-2R) is expressed in a highly tissue-specific manner, predominantly in the gastrointestinal tract and brain (Estall and Drucker, 2003). In contrast, the GLP-1 receptor (GLP-1R) has a more widespread distribution and is expressed in a number of tissues, including the pancreas, intestine, stomach, central nervous system, heart, pituitary, lung and kidney (Wei and Mojsov, 1996; Merchenthaler *et al*., 1999; Satoh *et al*., 2000). Both GLP-1 and GLP-2 are rapidly inactivated in the circulation as a consequence

of amino-terminal cleavage by the ubiquitous protease enzyme dipeptidyl peptidase-IV (DPP-IV). The inhibition of DPP-IV activity or the development of DPP-IV resistant glucagon-like peptide analogues offers additional systematic research options.

Compared to mammalian species, practically there has been relatively little investigations of the proglucagon-derived peptides in birds. GLP-1 appear to function similarly in both birds and mammals (Tachibana *et al*., 2004; Tachibana *et al*., 2006; Honda *et al*., 2007; Shousha *et al*., 2007; Tachibana *et al*., 2007). It is hypothesized that feed intake is one of the signals affecting GLP-1 and GLP-2 secretion in birds. GLPs actively participate in physiological body processes of chicken through direct or indirect mechanisms. Thus, the purpose of this dissertation was to clarify the mechanisms of GLPs secretion in chicken intestine using morphological techniques covering colocalization of GLPs, effects of quantity as well as quality (protein) of feed intake on intestinal L-cells. There are five experiments conducted under the research protocol presented in this dissertation with the following chapters:

Chapter II

Colocalization of GLP-1 with GLP-2 in L-Cells of the Chicken Intestine

II. 1. Introduction

GLP-1, a meal-induced gut hormone (Elliott *et al*., 1993; Balkan, 2000), has many important physiological actions, such as accentuation of glucose-dependent insulin release, inhibition of glucagon secretion and increase of pancreatic B cell growth (Fridolf *et al*., 1991; Nauck, 1998; Drucker, 2006). Moreover, it has other important biological functions; e. g., reduction of food intake (Furuse *et al*., 1997; Balkan, 2000), deceleration of gastric emptying (Nauck, 1998; Schirra *et al*., 2006) and intestinal motility (Tolessa *et al*., 1998a, b). Studies in chicken intestine have shown that GLP-1-immunoreactive cells are mainly distributed in the whole jejunum and ileum, and rarely found in ascending duodenum, but not in other intestinal regions (Hiramatsu *et al*., 2003, 2005). Pirone *et al.* (2012) showed similar findings in the pheasant gastrointestinal tract. GLP-2 is an intestinotrophic peptide hormone and perform many important functions, such as stimulation of the intestinal growth and inhibition of apoptosis in the intestinal crypt compartment (Baggio and Drucker, 2004). This hormone is derived from proglucagon in intestinal L-cells (Burrin *et al*., 2003; Drucker, 2006), so that these two GLPs are colocalized in the same secretory granule of the mammalian intestinal L-cells (Varndell *et al*., 1985). This colocalization, however, is not demonstrated in the chicken intestine. Colocalization data of GLP-1 and GLP-2 may present important information to understand the mechanisms of GLPs secretion in chicken. The present study aimed to clarify the colocalization of GLP-1 with GLP-2 in chicken intestinal L-cells using double immunofluorescent technique.

II. 2. Materials and Methods

2. 1. *Experimental birds*

White Leghorn chicks (*Gallus gallus*) (n=12, male) at one day of age were commercially obtained from Komatsu Shukeijyo (Matsumoto, Japan) and reared in our laboratory given a commercial diet (CP 17%) and water *ad libitum*, under continuous lighting for 24 hr up to

one week of age. Chicks were treated in accordance with the "Guideline for Regulation of Animal Experimentation (1997)" of Faculty of Agriculture, Shinshu University.

2. 2. *Tissue samples*

Chicks were perfused with a mixture of 4% paraformaldehyde and 0.01% glutaraldehyde in phosphate buffer (pH 7.6) following 0.75% NaCl solution under anesthesia with diethyl ether. Proximal and distal portions of ileum were immediately dissected out as tissue samples and then immersed in the same perfusate for 5 hr at 4℃. Tissue samples were cut into small blocks and pieces and embedded in paraffin wax according to standard procedure.

2. 3. *Double-staining immunofluorescent technique*

Paraffin sections cut at 5μm thickness were treated with 2.5% normal donkey serum (IHR-8135, ImmunoBioScience, Mukilteo, Washington, USA) for 20 minutes and incubated with mouse monoclonal antibody against synthetic human GLP-1(aa 7-36 amide) (diluted to 1:2,000, A6104.1, Immunodiagnostik, Bensheim, Germany) for 24 hr. After several washing with phosphate buffered saline (PBS), paraffin sections were subsequently incubated with rabbit anti-human (Arg^{34})-GLP-2 serum (1:500, H-028-14, Phenix Pharmaceuticals, Burlingame, California, USA) for 24 hr. Incubation of sections with the cocktail of FITC-labeled donkey anti-mouse IgG (1:100, GTX85337, GeneTex, Irvine, California, USA) and rhodamine-labeled donkey anti-rabbit IgG (1:100, 611-700-127, Rockland Immunochemicals, Gillbertsville, Pennsylvania, USA) was carried out for 3 hr. Sections were coverslipped with aqueous mounting medium (PermaFluor, Thermo Fisher Scientific, Fremont, California, USA), observed and photomicrographed under a fluorescent microscope (AxioImager, Zeiss, Göttingen, Germany). All incubations were carried out in the moisture chamber at room temperature. The specificities of primary antibodies used in this study were documented by the manufacturers and in previous reports (Eissele *et al*., 1992; Theodorakis *et al*., 2006). Primary antibodies against GLP-1 and GLP-2 mentioned above were preabsorbed with chicken/common turkey GLP-1(7-36) amide

(10^{-6} M/ml, H-5824, Bachem, Bubendorf, Switzerland) at 4°C for 24 hr and then used for confirming the specificities of them. Preabsorbed GLP-1 monoclonal antibody showed the negative reaction, but preabsorbed GLP-2 antiserum did the specific immunoreactivity. Moreover, single immunofluorescent staining was carried out for each primary antibody and compared with double immunofluorescent staining. Negative control sections were incubated with the normal donkey serum instead of the specific primary antibody or in the absence of the primary antibody. They showed negative immunoreactivity. Paraffin sections from rat ileum were used for positive control sections and showed the specific immunoreactivity for each primary antibody. The specificity of the secondary antiserum was controlled by the omission of the primary antibody. No cross reactivity of the secondary antiserum was observed. Immunoreactive cells were identified as well as categorized as either red (stained with one of the monoclonal antibody) and green fluorescence (stained with one of the polyclonal antibodies).

II. 3. Results

Immunofluorescent staining for GLP-1 and GLP-2 revealed that endocrine cells showing immunoreactivity for each peptide were scattered in the villous epithelium and crypt of the chicken ileum. Both immunoreactive cells showed the similar localization at the same cells in crypts of chicken ileum indicated by double immunofluorescent staining (Fig. 1, arrows). L-cells showing immunoreactivity for both GLP-1 and GLP-2 were observed in the whole ileum. They were located in crypts and epithelium of lower part of intestinal villi and showed comma-like or flask-like shape (Fig. 2a, b, arrows). These cells had cytoplasmic processes and were in contact with the intestinal lumen. L-cells showing immunoreactivity for only GLP-1 were located in epithelium of lower and middle parts of intestinal villi (Fig. 2c, d, arrowhead). All GLP-2-immunoreactive cells showed immunoreactivity for GLP-1.

II. 4. Discussion

The present study clarified the colocalization of GLP-1 with GLP-2 in the same L-cells of the chicken small intestine. L-cells in the chicken small intestine are characterized by microvilli covering their apical surface (Nishimura *et al*., 2013). Previous studies at the light microscopic level have reported the morphological features and the distributional pattern of GLP-1-immunoreactive cells in the chicken small intestine (Hiramatsu *et al*., 2003, 2005). According to these studies, GLP-1-immunoreactive cells show comma-like or flask-like shape and are in contact with the intestinal lumen by their apical cytoplasmic process. These findings indicate that L-cells are namely open-type of endocrine cells and receive chemical signals in the intestinal lumen, such as digests of ingested meal. In fact, GLP-1 is released in response to feed intake and exerts its physiological actions in mammals (Elliott *et al*., 1993; Balkan, 2000).

Human GLP-2 is a gastrointestinal hormone with 33% sequence homology to glucagon and shows the intestinotrophic endocrine/paracrine actions (Thulesen, 2004). This peptide is derived from proglucagon by its posttranslational processing and secreted from intestinal L-cells. Thus, two forms of GLP, GLP-1 and -2, are synthesized from the same precursor and secreted from the same cells in the mammalian intestine. Immunocytochemical findings provide the prediction of co-secretion of GLP-1 with GLP-2 from the same L-cells also in the chicken small intestine (Nishimura *et al*., 2013). A double immunofluorecent study demonstrated that L-cells showing the colocalization of GLP-1 with GLP-2 were mainly located in crypts and epithelium of lower part of villi of chicken small intestine. Moreover, L-cells showing immunoreactivity only for GLP-1 were observed in epithelium of lower and middle parts of villi. These findings hints that most probably both GLP-1 and GLP-2 secrete simultaneously at first and then only GLP-1 to perform their respective activities. Considering these findings at light microscopic level, it is possible that GLP-1 and -2 are co-stored in the same secretory granules, but secreted from the same L-cells

separately. Hiramatsu *et al*. (2005) previously reported that GLP-1 immunoreactive cells abundantly occur in the chicken small intestine, and the present experiment showed the same manner in case of GLP-2. It is, however, highly probable to coincident of these two cells in crypts of different regions of the chicken small intestine. Colocalization of GLP-1 and GLP-2 immunoreactivities has been observed within single enteroglucagon cell secretory granules in human gut (Varndell *et al*., 1985). The present study proved the simultaneous colocalization of two antigens, GLP-1 and GLP-2, on a single tissue section. Thus, colocalization of GLP-1 and -2 provides additional information for future systematic in detailed studies on the physiological roles of gastrointestinal peptides in the gastrointestinal tract of the chicken. In conclusion, GLP-1 and GLP-2-immunoreactive cells are colocalized in the chicken small intestinal L-cells and immunoreactivity for GLP-1 only also demonstrated.

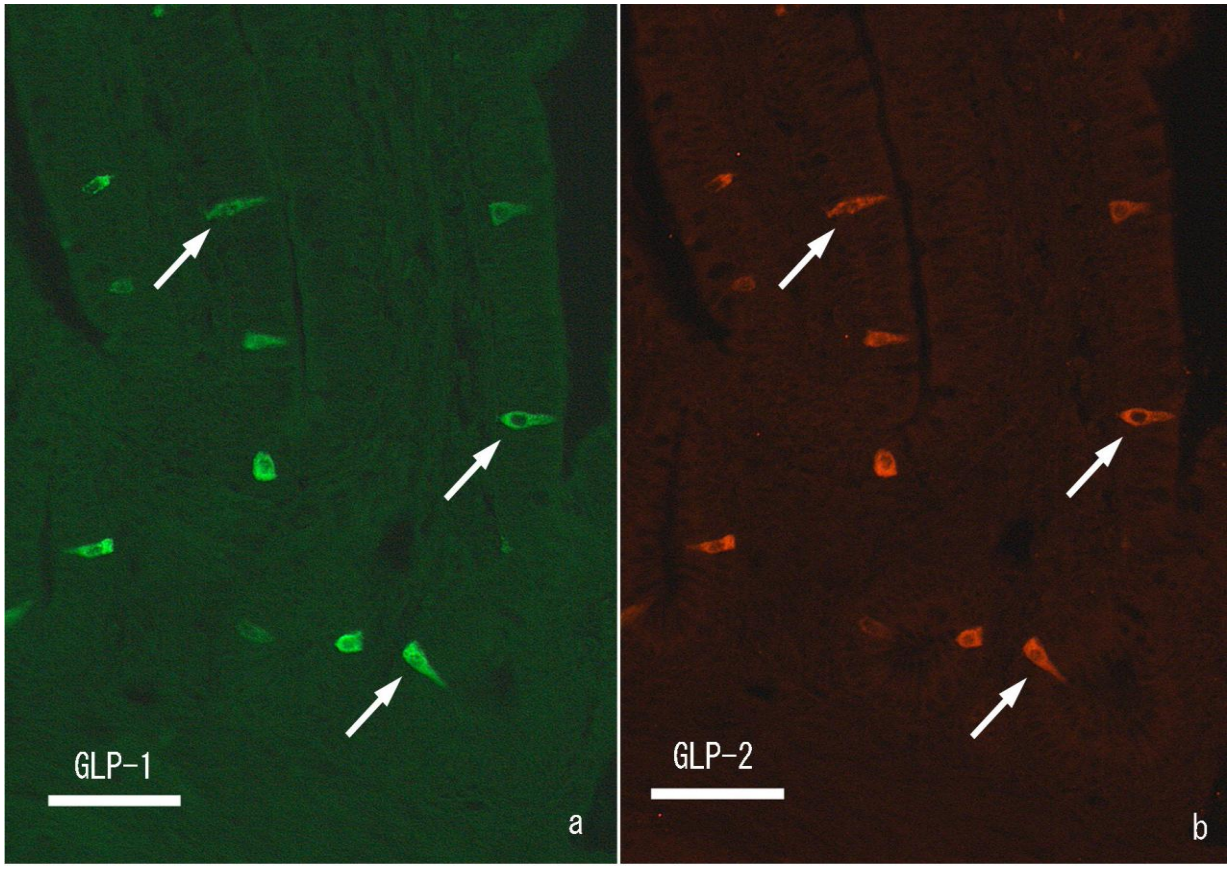

Fig. 1 Double immunofluorescent staining for GLP-1 (**a**) and GLP-2 (**b**). Many endocrine cells showing immunoreactivity for both GLP-1 and GLP-2 (arrows) were observed in the epithelium of intestinal villi in the chicken ileum. Bar: 50 μm.

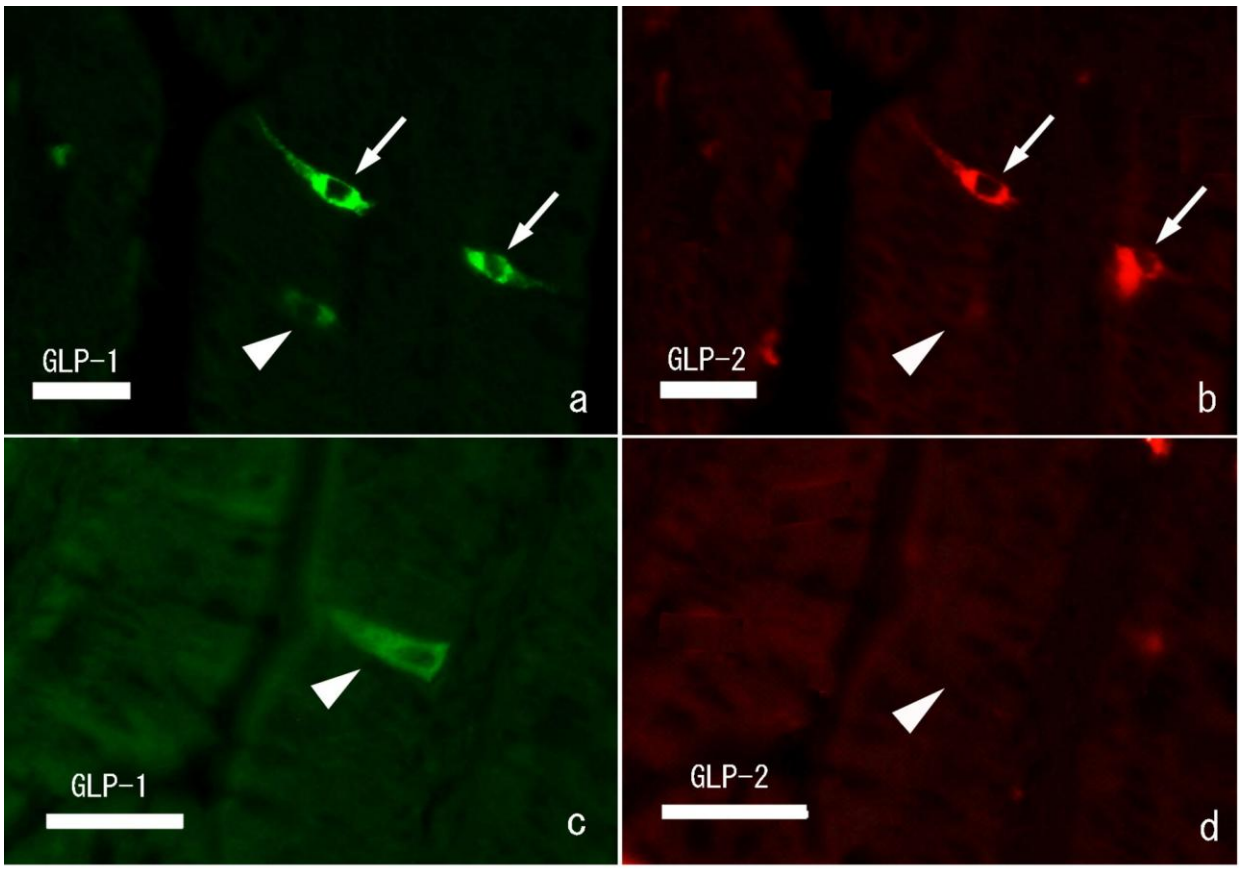

Fig. 2 Double immunofluorescent staining for GLP-1 (**a, c**) and GLP-2 (**b, d**). Arrows indicate endocrine cells showing immunoreactivity for both GLP-1 and GLP-2. Arrowheads indicate endocrine cells showing only GLP-1 immunoreactivity. Distal ileum. Bar: 20 μm.

II. Summary

Colocalization of GLP-1 with GLP-2 in L-cells was investigated in the chicken ileum using the double immunofluorescent technique. GLP-1 and GLP-2-immunoreactive cells are co-stored in the chicken small intestinal L-cells and immunoreactivity for GLP-1 only also demonstrated. L-cells showing immunoreactivity for both GLP-1 and GLP-2 were distributed in the whole ileum. They showed comma-like or flask-like shape and were located in crypts and epithelium of lower part of intestinal villi. L-cells showing GLP-1-immunoreactivity only were found in epithelium of lower and middle parts of intestinal villi. These findings hints that most probably both GLP-1 and GLP-2 secrete simultaneously at first and then only GLP-1 to perform their respective activities. This study advances new morphological data about the endocrine system in the chicken small intestine.

Chapter III

Influence of Restricted Feeding on GLPs

III. 1. Influence on GLP-1-immunoreactive cells in the chicken small intestine

III. 1. 1. Introduction

GLP-1 is a 36-amino acid peptide derived by specific post-translational proteolytic cleavage and other enzymatic modifications of proglucagon and secreted from L-cells in the intestine (Drucker, 2006; Richards and McMurtry, 2009). This meal-induced gut hormone (Elliott *et al*., 1993; Balkan, 2000) has many important physiological actions such as the accentuation of glucose-dependent insulin release, the inhibition of glucagon secretion and the increase of pancreatic β-cell growth (Fridolf *et al*., 1991; Nauck, 1998; Drucker, 2006). Moreover, GLP-1 has other important biological functions; e.g. reduction of food intake (Furuse *et al*., 1997; Balkan, 2000) and deceleration of gastric emptying (Nauck, 1998; Schirra *et al*., 2006) and intestinal motility (Tolessa *et al*., 1998a, b). Immunohistochemical and morphometrical studies in the chicken intestine have shown that the immunoreactive cells against GLP-1 antiserum are mainly distributed in the whole jejunum and ileum, and rarely found in ascending duodenum, but not in other intestinal regions (Hiramatsu *et al*., 2003; 2005). These findings suggest that GLP-1 plays an important role in the regulation of activities of the chicken small intestine. More detailed studies on this topic are, however, needed to uncover the role of GLP-1 hormone in the regulation of the intestinal activities; e. g., the effect of feeding manner and nutrients on GLP-1 secretion, mechanism of GLP-1 secretion and the interaction between GLP-1 and the enteric nervous system have to be elucidated.

In the present study, it is aimed to clarify the influence of restricted feeding, to understand the effects of quantity of feed intake on GLP-1-immunoreactive endocrine cells in the chicken small intestine by using immunohistochemical and morphometrical methods. The role of GLP-1 in the regulation of intestinal motility is also discussed in the present study.

III. 1. 2. Materials and Methods

1. 2. 1. *Experimental birds*

White Leghorn chicks (*Gallus gallus*) (n=15, male) were commercially obtained from Komatsu Shukeijyo (Matsumoto, Japan) and reared in our laboratory under continuous lighting for 24 hr, up to 6 weeks of age (average body weight=580.8g). The birds were given a commercial diet (CP 17%) and water *ad libitum*. Three days were allowed to acclimatize the birds with the feeding pattern before the initiation of experimental feeding in separate cages. The daily feed intake of each chicken during the adapting period was measured and then the daily feed supplies for chickens of restricted groups were calculated. After adapting period, chickens were randomly divided into three groups (100%: control, 50% and 25% feed supply groups) of five chickens each. During the experimental period of seven days, daily feed supply to 50% and 25% feed supply groups were restricted by 50% and 75% that of the adapting period (Table 1). Chickens in the control group were fed by the same manner as that during the adapting period. All groups were kept under standardized temperature, humidity and controlled light condition. Daily feed intake and body weight gain or loss of each chicken during the experimental period was measured (Table 1). Chickens were treated in accordance with the "Guideline for Regulation of Animal Experimentation (1997)" of Faculty of Agriculture, Shinshu University.

1. 2. 2. *Tissue samples*

After the experimental period, chickens were sacrificed by decapitation under anesthesia with sodium pentobarbital. Intestinal tissue samples about 2 cm long were rapidly dissected out from each bird, washed with 0.75% NaCl solution and immersed in Bouin's fluid overnight at room temperature. Samples were collected from proximal and distal regions of jejunum and ileum.

1. 2. 3. *Immunohistochemistry*

Tissue samples were embedded in paraffin wax according to standard procedures. Sections were cut at 5μm thickness and used for the detection of GLP-1 by immunohistochemical technique. Streptavidin-biotin method (Guesdon *et al*., 1979) was applied to detect GLP-1-immunoreactive cells according to the procedures previously described by Hiramatsu and Ohshima (1995). Rabbit antiserum against synthetic GLP-1(1-19) conjugated to bovine serum albumin (Affiniti Research Products, UK, No.GA1176, diluted to 1:2000) was used as the primary antibody in this study. This antiserum dose not cross-react with other proglucagon-derived peptides (Tachibana *et. al.*, 2005).

1. 2. 4. *Morphometry*

Morphometrical analysis was carried out according to the previously described method (Hiramatsu *et al*., 2005). To evaluate the frequency of occurrence of GLP-1-immunoreactive cells in each intestinal region, the immunoreactive cells with clearly identifiable nuclei were counted, and the area of the mucosal layer was measured. The cell number per area of the mucosal layer (cells/mm^2 ± SD) was then calculated. This quantification was carried out using a computerized image analysing system (KS400, ZEISS, Göttingen, Germany). Twenty areas were measured in each intestinal region from each bird. One hundred areas in total were measured in each intestinal region from five chickens in each group. Statistical analyses were performed to assess the differences in the frequency of GLP-1-immunoreactive cells among three groups at each intestinal region using Tukey's method (Yanai, 2011).

III. 1. 3. Results

1. 3. 1. *Weight gain of chickens on restricted diets*

Average feed intake (g/d/head) and body weight gain (g/head) of three groups during the experimental period are summarized in Table 1. Body weight gains were negatively balanced in 50 and 25% feed supply groups; -18.6g and -105.2g, respectively.

1. 3. 2. *Morphology of GLP-1-immunoreactive cells*

Endocrine cells showing immunoreactivity for GLP-1 antiserum were observed in the whole jejunum and ileum of three groups (Fig. 3a-c). In the control group, GLP-1-immunoreactive cells were mainly distributed in crypts and epithelium of the lower part of intestinal villi (Fig. 3a). In 50 and 25% feed supply groups, however, enteroendocrine cells showing GLP-1 immunoreactivity tended to be more observed in epithelium of the middle part of intestinal villi and to be fewer in crypts (Fig. 3b, c). GLP-1-immunoreactive cells had pyramidal or spindle-like shape in villous epithelium (Fig. 4a-c) and comma-like shape in crypts (Fig. 4d-f) and were in contact with the intestinal lumen with their cytoplasmic process. There was no obvious difference in shape of GLP-1-immunoreactive cells among three groups.

1. 3. 3. *Distribution of GLP-1-immunoreactive cells*

GLP-1-immunoreactive cells showed similar distributional pattern to that previously described in the chicken small intestine: their frequency of occurrence was higher in the distal region than the proximal region of the small intestine. There were, however, significant differences in their frequency of occurrence at all intestinal regions between the control and 25% feed supply group (Fig. 5). Frequencies of occurrence of GLP-1-immunoreactive cells in the control and feed restricted two groups were summarized in Table 2. There were significant differences in the frequency of occurrence of GLP-1-immunoreactive cells between the control and 25% feed supply group at each intestinal region ($p<0.01$), but not between the control and 50% feed supply group.

III. 1. 4. Discussion

The present study demonstrated that the restricted feeding had an influence on the frequency of occurrence of GLP-1-immunoreactive cells in the chicken small intestine. There were significant differences in the frequency of occurrence of GLP-1-immunoreactive cells in the chicken small intestine between control and 25% feed supply

groups. This result indicates that larger number of L-cells containing GLP-1 in their cytoplasm are located in the intestinal epithelium of restricted chickens than control ones. These results agreed with the findings of Takahashi *et al*. (1992) who reported a significant decrease in the plasma levels of GLP-1 immunoreactivity during 5 days of fasting in rat. The reason for this might be due to a lack of nutritional triggers to activate the secretory mechanism for GLP-1, and therefore large numbers of cells are retained intact showing immunoreactivity or alternatively, more cells could be produced as a response to a demand for more GLP-1 to be secreted to keep the blood glucose levels at a normal functioning range. He also showed that the pancreatic contents of proglucagon-derived peptides in normal fed and starved rats are more or less same and no significant change in the posttranslational processing of proglucagon is detected during starvation. So there is a possibility to increase in visible GLP-1 secretory cell numbers in the intestine, rather than the pancreas. Juhl *et al*. (2002) also demonstrated that GLP-1 derivative effectively reduces fasting in man by modifying insulin secretion, delaying gastric emptying, and suppressing glucagon secretion to meet up the emergency situations. It is generally assumed that number of endocrine cells present in the intestinal tract is proportional to their regulatory characteristics as well as repair and renewal potential.

L-cells in the chicken small intestine are ‘open-type’ endocrine cells which have an apical cytoplasmic process accessing the intestinal lumen like enteroendocrine cells containing other hormones (Hiramatsu *et al*., 2003; 2005). The ‘open-type’ enteroendocrine cells are considered as primary candidates for intestinal sensors (Breer *et al*., 2012). Several studies showed that ingested nutrients such as carbohydrates, proteins and fatty acids had several influences on L-cells in the mammalian intestine (Hansen *et al*., 2004; Cani *et al*., 2007; Karhunen *et al*., 2008; Yoder *et al*., 2009). Thus ingested feed is the major signal which stimulates the GLP-1 release from L-cells as mentioned previously (Elliot *et al*., 1993; Balkan, 2000; Karhunen *et al*., 2008). In fact, the frequency of occurrence of GLP-1

immunoreactive cells is increased, specially in ileum of the chickens fed low amount of feed, most probably, due to lack of nutritional response controlling the motility of intestinal lumen. Moreover, this factor also causes the disruption in the normal physiological phenomena of the birds' resulting underweight in comparison with control group. These results indicate that the quantity of ingested feed had an influence on endocrine cells showing immunoreactivity for GLP-1 in the chicken small intestine.

Many regulatory peptides from endocrine cells in the mucosal epithelium of the alimentary tract as well as from neurons in the enteric nervous system are involved in the control of the intestinal motility (Dockray and Walsh, 1994). GLP-1 secreted from L-cells stimulates insulin release from pancreatic B cells (Fridolf *et al*., 1991; Nauck, 1998) and had many other physiological actions including the inhibition of gastric emptying (Drucker, 2006). Tolessa *et al*. (1998a, b) demonstrated the inhibitory effect of GLP-1 on small bowel motility. Yoder *et al*. (2009) showed that GLP-1 was secreted by dietary lipid to reduce intestinal motility and enhance the proximal fat absorption. GLP-1-containing L-cells show a continuous increase of their number from the proximal to the distal portion of small and large intestine of mammals (Eissele *et al*., 1992; Damholt *et al*., 1999). In the avian alimentary tract L-cells are common in the distal small intestine (Hiramatsu *et al*., 2003; 2005; Pirone *et al*., 2012). It is possible that GLP-1secreted at the distal small intestine enhances the absorption of nutrients at the proximal portion by inhibiting the intestinal motility in the chicken alimentary tract. It is said that the inhibition of intestinal motility by GLP-1 comes out through an indirect effect via central and enteric nervous system (Tolessa *et al*., 1998a, b; Stanley *et al*., 2004). Dense network of pituitary adenylate cyclase-activating peptide (PACAP)-immunoreactive nerve fibres was found in the lamina propria of villi of chicken small intestine (Pirone *et al*., 2011). These findings indicate the close relationship between enteroendocrine cells in epithelium and nervous elements in the lamina propria. L-cells are probably one of primitive candidates for intestinal sensors in the

chicken small intestine. In the present study, the remarkable levels of GLP-1IR in the small intestine of restricted fed chickens demonstrate the potentiality of studying the secretory physiology of this hormone. In addition, the brain plays a highly critical role in the control of feeding behaviors of animals (Konturek *et al*., 2004). The gut is intimately connected with the actions of the brain via the release of peptides in the regulation of feeding. So these data raise the opportunity to explore the specialized signaling mechanisms of gut-brain interactions in governing this phenomenon and more systematic studies are required for its clarification.

In conclusion, the quantity of ingested food is one of signals which have an influence on the secretion of GLP-1 from the chicken small intestine. These results suggest the regulation of intestinal motility by GLP-1 with the regulation of pancreatic hormones.

Table 1. Average feed intake (g/d/head) and body weight gain (g/head) during the experimental period. Chickens of 50 and 25% feed supply groups were provided with feed at a reduction of 50 and 75% of that during adapting period. Body weight gains were negatively balanced in 50 and 25% feed supply groups; -18.5g and -105.2g, respectively.

Groups	**Feed intake (g/d/head) (mean ± SD)**	**Body weight gain (g/head) (mean ± SD)**
100%: Control	65.5 ± 2.1	159.8 ± 15.0
50% Feed supply	33.2 ± 2.1	-18.6 ± 17.4
25% Feed supply	16.4 ± 1.0	-105.2 ± 24

Table 2. Frequencies of occurrence of GLP-1-immunoreactive cells in proximal and distal regions of jejunum and ileum from the 100%: control, 50 and 25% feed supply groups (cells/mm^2 mucosal area, mean ± SD).

Groups (n = 5)	Jejunum		Ileum	
	Proximal	**Distal**	**Proximal**	**Distal**
100%: Control	2.33 ± 1.33	17.32 ± 5.56	24.89 ± 7.25	29.93 ± 8.90
50% Feed supply	5.19 ± 3.51	13.40 ± 7.74	23.68 ± 6.44	35.19 ± 6.48
25% Feed supply	9.02 ± 2.64*	31.23 ± 4.95*	42.65 ± 6.17*	46.81 ± 4.80*

*Significant difference versus the 25% feed supply and 100%: control group at proximal jejunum, and versus the control and 50% feed supply group at distal jejunum and ileum; $P < 0.01$.

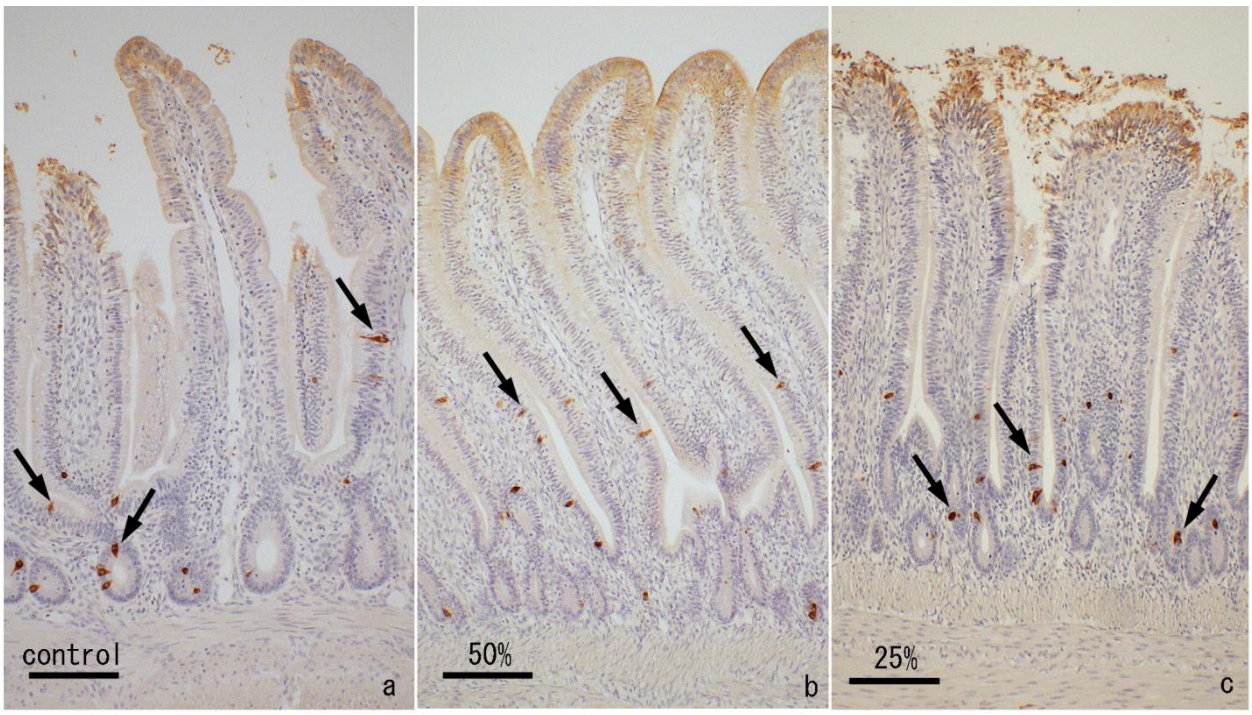

Fig. 3 GLP-1-immunoreactive cells (arrows) in the distal ileum from the control group (**a**), 50% feed supply group (**b**) and 25% feed supply group (**c**). Bar: 100 μm.

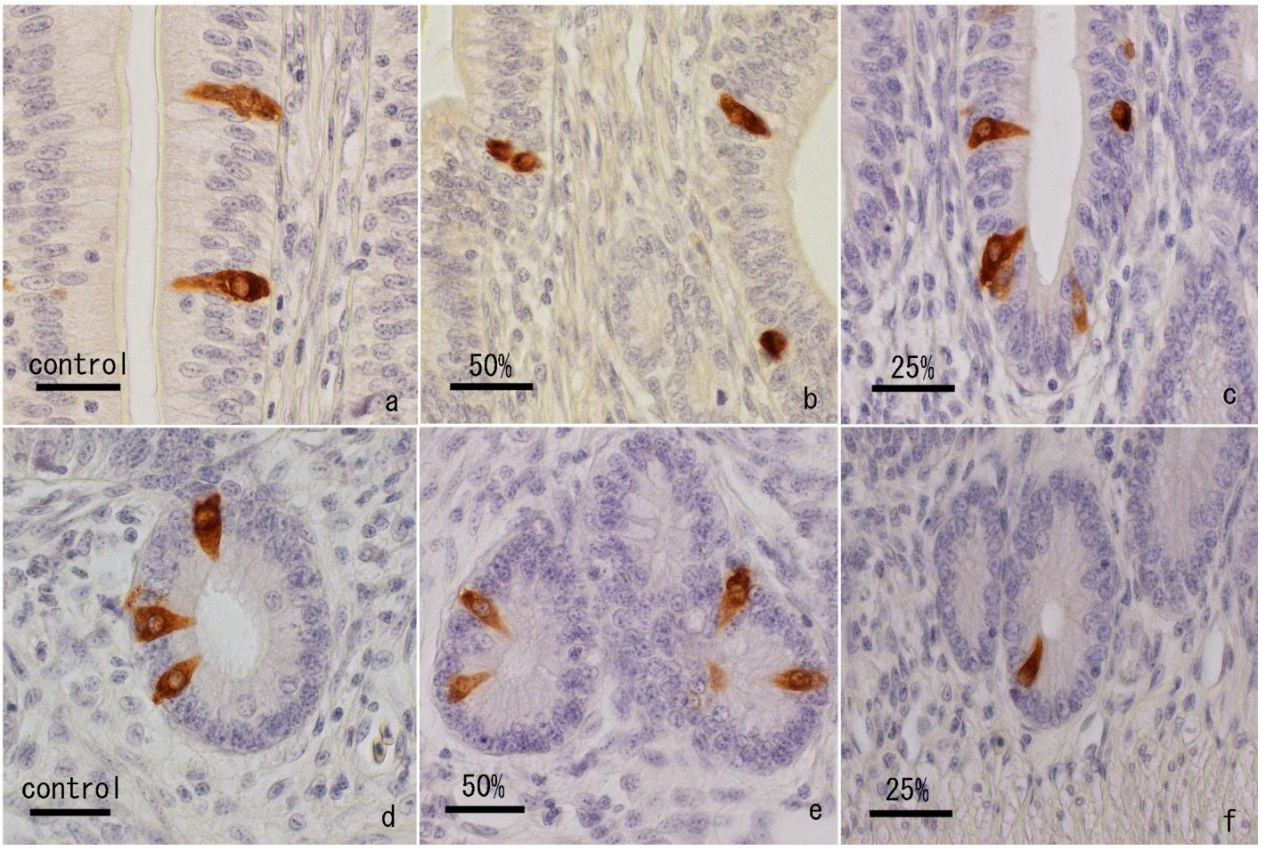

Fig. 4 High magnification views of GLP-1-immunoreactive cells in the distal ileum from the control group (**a, d**), 50% feed supply group (**b, e**) and 25% feed supply group (**c, f**). No obvious morphological differences are observed in villous epithelium (**a-c**) and crypts (**d-f**) among three groups. Bar: 20 μm.

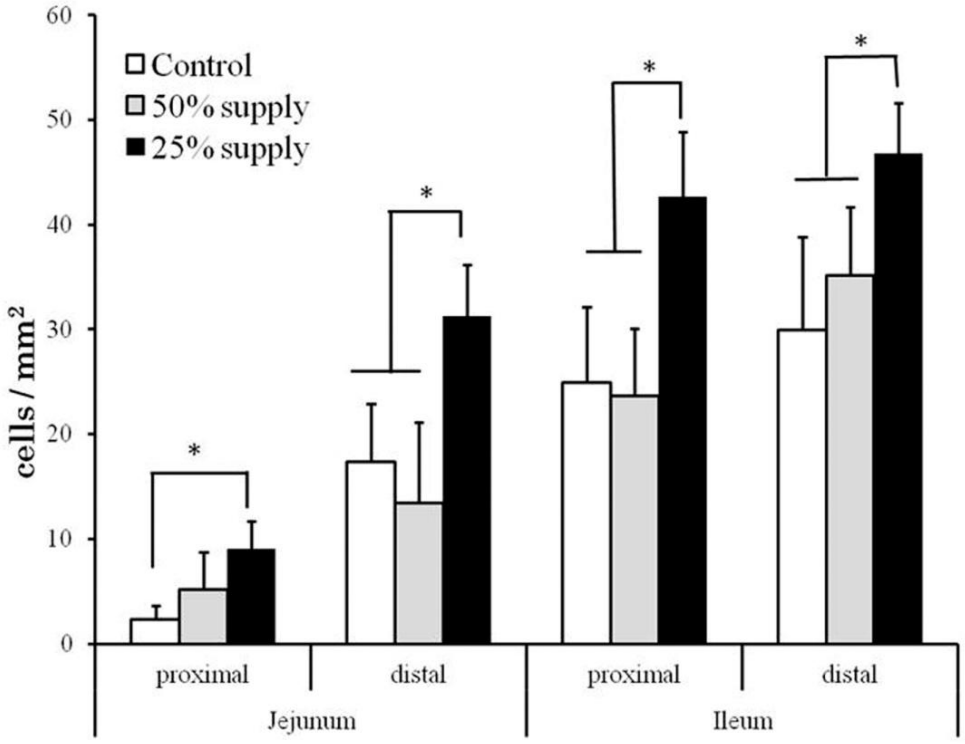

Fig. 5 Frequency of occurrence of GLP-1-immunoreactive cells in proximal and distal segment of jejunum and ileum from the control, 50% and 25% feed supply groups (cells/mm^2 mucosal area). Asterisks shows a significant difference in the same intestinal region ($p<0.05$, error bars: SD).

III. 2. Influence on GLP-2-immunoreactive cells in the chicken small intestine

2. 1. Introduction

GLP-2 is a 33-amino acid intestinotrophic peptide derived by specific post-translational proteolytic cleavage and other enzymatic modifications of proglucagon in the intestinal organelles of "open-type" endocrine L-cells in monogastric species (Lovshin and Drucker, 2000; Burrin *et al*., 2003; Drucker, 2005). This meal-induced gut hormone exerts diverse actions in the gastrointestinal tract including stimulation of intestinal growth, up-regulation of villus height, concomitant with crypt cell proliferation or crypt depth and reduced enterocyte apoptosis, enhance mucosal blood flow, and most importantly nutrient assimilation, decelerate gastric motility and secretion (Guan *et al*., 2006) and modulate food intake by providing a feedback signal to the brain (Brubaker, 2006). GLP-2 has activities of cytokines, enhancing cell differentiation, playing a role in tissue regeneration, and mediating cytoprotection (Drucker, 2003). It also plays a key role in intestinal glucose transport and decreases mucosal permeability. Studies have shown that GLP-2 had specific tropic effects on the intestine of humans, rodents and other mammals (Burrin *et al*., 2001). In contrast, there are no documented reports about the distributional pattern, and effects of restricted feeding on GLP-2- immunoreactive cells in the chicken intestine. Detailed study on this topic is, however, required to uncover the role of GLP-2 hormone in the control of the intestinal activities. In the present study, it is aimed to clarify the normal distribution of GLP-2-immunoreactive cells and the influence of restricted feeding on them in the chicken intestine using immunohistochemical and morphometrical techniques.

III. 2. 2. Materials and Methods

2. 2. 1. *Experimental birds*

White Leghorn chicks (*Gallus gallus*) (n = 15), weighing about 580.8g on an average, were used in this experiment. Chickens were maintained in our laboratory with proper and identical management following continuous lighting up to 6 weeks of age. The birds were

supplied with a balanced diet (CP 17%) and water *ad libitum*. Daily feed intake (g) of each chicken, feeding in separate cages, during the adapting period of 3 days were measured, and then the amount of daily feed requirement for chickens of restricted groups were calculated. Now the acclimatized chickens were randomly divided into three groups (100%: control, 50 and 25% feed supply groups) having five chickens each. During the experimental period of seven days, the chickens of control group were fed as it is, and 50 and 25% feed supply groups were provided with the daily feed at a reduction of 50 and 75% of that during adapting period each. Daily feed intake and body weight gain or loss of each chicken during the experimental period was measured (Table 1). Chickens were treated in accordance with the "Guideline for Regulation of Animal Experimentation (1997)" of Faculty of Agriculture, Shinshu University.

2. 2. 2. *Tissue samples*

After completion of experimental period, all the birds were killed by cervical dislocation under anesthesia with sodium pentobarbital. Intestinal tissue samples (about 2 cm) from duodenum, proximal and distal regions of jejunum, ileum and cecum, and colorectum were rapidly dissected out from each bird, washed with 0.75% NaCl solution and immersed in Bouin's fluid overnight at room temperature.

2. 2. 3. *Immunohistochemistry*

Following standard procedure the tissue samples were embedded in paraffin wax and sections were cut typically at 5μm thickness with a sliding microtome, rehydrated in progressively low concentrated ethanol baths followed by xylene. Immunohistochemical staining for GLP-1 proceeded relatively well without the treatment with antigen retrieval agent (Hiramatsu *et al*., 2003; 2005). But, in this case, immunohistochemical staining for GLP-2 showed very small numbers of endocrine cells. It is thought that the epitope of GLP-2 may be masked in intestinal L-cells, immunoreactivity for GLP-2 is difficult to detect by the ordinary procedure for immunohistochemical staining. So to retrieve antigens, the

sections were incubated with 0.5% antigen retrieval medium (citraconic anhydride, Immunosaver®, Nisshin EM Corporation, Tokyo, Japan) at 98°C for 45 min and rinsed with phosphate buffered saline (pH 7.6). Sections pretreated with 10% normal goat serum (No. 1105602A, Invitrogen, USA) were incubated with rabbit antiserum against the primary antibody synthetic GLP-$2_{(1-34)}$ conjugated to bovine serum albumin (Phoenix Pharmaceuticals Inc. USA, H-028-14, diluted to 1:2000) at room temperature overnight. To identify GLP-2 immunoreactive cells, the preparation was visualized using HRP-labeled streptavidin-biotin method (Guesdon *et al.*, 1979) and DAB- as a chromogen, previously described by Hiramatsu and Ohshima (1995). After counterstaining with Mayer's hematoxylin, sections are mounted and coverslipped to observe under light microscope. The specificity of primary antibody used in this study was documented by the manufacturers and in previous report (Nishimura *et al*., 2013). Primary antibody against GLP-2 mentioned above was preabsorbed with chicken GLP-2(1-34) and showed the specific immunoreactivity.

2. 2. 4. *Morphometry*

The morphometrical analyses was carried out following a method described by Hiramatsu *et. al.* (2005). We only counted the cells with clearly detectable nuclei and the area of the mucosal layer was also measured accordingly. Then cell number per area of the mucosal layer (cells/mm^2) was calculated to find out the frequency of occurrence of GLP-2- immunoreactive cells in each intestinal region. This computation was performed using a computerized image analyzing system (KS400, ZEISS, Göttingen, Germany). Twenty areas were measured in each intestinal region from each bird, i. e., 100 areas in total from 5 chickens of each group. Tukey's method (Yanai, 2011) of statistical analyses were followed to assess the differences in the frequency of occurrence of GLP-2-immunoreactive cells among three groups. $P \leq 0.05$ was set as the level of significance.

III. 2. 3. Results

2. 3. 1. *Normal distribution and morphology of GLP-2-immunoreactive cells*

Endocrine cells showing immunoreactivity for GLP-2 antiserum were identified in the whole jejunum and ileum, but not in other intestinal regions. The cells were most densely distributed in the distal ileum, moderately in both proximal ileum and distal jejunum followed by proximal jejunum. The majority of GLP-2-immunoreactive cells were localized on crypts with lesser numbers present in the villus epithelium (Fig. 6a). Most of the cells were pyramidal or spindle-like shape in the villous epithelium (Fig. 7a) and comma-like shape in crypts (Fig. 7d) with an apical cytoplasmic process that established contact with the intestinal lumen. The frequencies of occurrence of GLP-2- immunoreactive cells in the proximal and distal ileum were 23.5 ± 4.7 and 34.6 ± 4.9, respectively (cell numbers per mucosal area : cells/mm^2, mean ± SD), and in jejunum these were 14.7 ± 2.3 and 19.8 ± 2.3, respectively (Fig. 8). There were significant differences among the adjacent regions of jejunum and ileum ($p<0.05$). The frequencies of occurrence of GLP-2 immunoreactive cells were gradually increased with the advancement to the distal region of the small intestine.

2. 3. 2. *Effect of feed restriction on GLP-2 morphology*

Endocrine cells showing immunoreactivity for GLP-2 antiserum were observed in the whole jejunum and ileum of three groups (Fig. 6a-c). In the control group, GLP-2-immunoreactive cells were mainly distributed in crypts and epithelium of the lower part of intestinal villi (Fig. 6a). In 50% and 25% feed supply groups, however, enteroendocrine cells showing GLP-2-immunoreactivity tended to be more observed in epithelium of the middle part of intestinal villi and to be fewer in crypts (Fig. 6b, c). GLP-2-immunoreactive cells had pyramidal or spindle-like shape in villous epithelium (Fig. 7a-c) and comma-like shape in crypts (Fig. 7d-f) and were in contact with the intestinal lumen with their

cytoplasmic process. There was no obvious difference in shape of GLP-2-immunoreactive cells among three groups.

2. 3. 3. *Effect of feed restriction on GLP-2 cell number*

Fig. 8 shows that the number of GLP-2-immunoreactive cells were lowest in control group, medium in 50% and highest in 25% feed supply group at each intestinal region examined in this study, that is, increased significantly ($p<0.05$) with the advancement of restricting the amount of feed supply. This result indicate that quantity of feed intake is one of the signals influencing the secretion of GLP-2 from L-cells in the chicken intestine.

III. 2. 4. Discussion

Previous experiments showed that immunohistochemical staining for GLP-1 proceeded relatively well without the treatment with antigen retrieval agent (Hiramatsu *et al*., 2003; 2005). In case of GLP-2, ordinary staining showed very small numbers of endocrine cells. Masking by fixative may be the main reason for this, forming methylene bridges between proteins, which can hinder epitope recognition by the primary antibodies. The heat-induced epitope retrieval method is the most common approach to expose the antigen epitopes in preparation for immunohistochemical staining. If this step is not performed, the antibodies will not have complete access to the tissue and will be unable to bind to the correct epitopes. Keeping this phenomenon in mind, antigen retrieval medium (citraconic anhydride) was applied to ensure identical as well as high quality antigen unmasking without damaging any cell/tissue morphology and molecules. Antigen retrieval leads to satisfactory results of immunohistochemical staining in paraffin-embedded tissue for a great number of antibodies tested (Yamashita, 2007; Shi and Taylor, 2010; Taylor *et al*., 2010).

In the present study, it became clear that there was a significant difference in the distributional pattern of GLP-2-immunoreactive cells between jejunum and ileum of the chicken. In other words, immunoreactive cells for GLP-2 were mainly observed in the

crypts of distal ileum followed by proximal jejunum. Saito *et al*. (1989) investigated that enteroglucagon-immunoreactive cells are distributed in the whole intestine with differing distribution patterns, nil in duodenum and most dominant in colorectum of the domestic pigeon, because the migration of enteroendocrine cells as well as other types of epithelial cells occurs along the crypt column (Tsubouchi, 1981). The shapes of the cells found in this study are similar with the findings of Hiramatsu *et al*. (2003) who indicated the spindle-like cells in the gastrointestinal tract of chicken and ostrich. It may be concluded that chicken intestine is capable of synthesizing, secreting and responding to GLP-2. And the highest frequency of occurrence of these cells is in the posterior part of the ileum.

The number of chicken intestinal GLP-2 cells per mm^2 are somewhat higher in comparison with GLP-1 (Table 3). This considerable discrepancies may be due to treatment of GLP-2 preparation with antigen retrieval. Ordinary procedure of immunohistochemical staining without antigen retrieval showed well immunoreactivity for GLP-1 but not GLP-2. The target organs for GLP-1 and GLP-2 are distinctive, β-cell of pancreas (islets of Langerhans) and intestinal epithelium, respectively that most probably have great deals in their numbers. Irwin and Wong (1995) showed that the chicken intestinal proglucagon mRNA spliced into one or more exons which encode GLP-2. In case of GLP-1, the cells are located more or less evenly throughout the whole crypts and villi. On the contrary, GLP-2 cells primarily positioned in the crypts and lower base of the villi. Perhaps, the reason lying behind this fact is variations in synthesis, secretion and purposes of this two hormones. Nishimura *et al*. (2013) showed that GLP-1 and GLP-2 are colocalized in chicken intestinal L-cells and they also found L-cells showing immunoreactivity for only GLP-1 were located in epithelium of lower and middle parts of intestinal villi. This findings may hint two types of secretory pattern of GLPs in the chicken small intestine: (i) at first GLP-2 secretes to maintain the total integrity of intestinal epithelial cells and then GLP-1 for incretin hormone functionality, (ii) in the beginning, both GLP-1 and -2 secrete simultaneously as

they are ontogenetically correlated and co-stored in the same L-cells, and later on only GLP-1 that lying solely and have no linkage with GLP-2. In human GLP-2 is co-secreted with GLP-1 from the intestinal enteroendocrine L-cells with the presence of luminal nutrients being the primary stimulus for secretion (Jeppesen *et al*., 2001). These findings are further supported by an earlier report by Ørskov *et al*. (1986) who mentioned GLP-1 and -2 secrete separately from pig small intestine.

Many regulatory peptides from endocrine cells in the mucosal epithelium of the alimentary tract as well as from neurons in the enteric nervous system are involved in the control of the intestinal motility (Dockray and Walsh, 1994). GLP-2 secreted from L-cells is one of such peptides that stimulates the expansion of the villous epithelium contributing both increased crypt cell proliferation and decreased enterocyte apoptosis (Holst, 2000). Comparing with GLP-1, GLP-2 was recognized recently, during the mid to late 1980s (Kieffer and Habener, 1999). The present study showed the detailed distribution of GLP-2 immunoreactive cells found in the mucosal epithelium of the chicken small intestine; a continuous increase of these cells was found from the proximal jejunum to the distal ileum showing the highest density. The regional distribution and relative frequency of gastrointestinal endocrine cells has been found to vary with the animal species and feeding habits (Solcia *et al*., 1975). There is the species differences in the distributional pattern of enteroglucagon-containing cells in the avian gastrointestinal tracts. Enteroglucagon-immunoreactive cells were detected in the chicken small intestine in increasing numbers forwards the ileum (Yamanaka *et al*., 1989). Many investigators, however, described the endocrine cells and their respective regulator peptides in the gastrointestinal tract of other vertebrates, such as birds (Rawdon and Andrew, 1981; Bezuidenhout and Van Aswegen, 1990; Nascimento *et al*., 2007), reptiles (Ku *et al*., 2001), fish (Pan *et al*., 2000), and mammals (Ørskov *et al*., 1986; Ku *et al*., 2004a, b). Enteroendocrine cells have been reported to be most numerous distally (rectum) in other species as well, including man

(Cristina *et al*., 1978; Kitamura *et al*., 1982; Calingasan *et al*., 1984). The secretion profile for GLP-2 is biphasic, with an early peak approximately 30 min after nutrient ingestion and a second, more prolonged peak at 60-120 min (Xiao *et al*., 1999). Studies in animals have demonstrated that nutrients reach in the proximal gut section of the intestine within the first 30 min after ingestion and the stimulus for the early postprandial peak of peptide YY (PYY) is mediated indirectly through a neuroendocrine pathway, whereas direct contact of luminal nutrients with L-cells is likely to induce the later peak of secretion (Fu-Cheng *et al*., 1997). Same consideration may be applicable in case of GLP-2-immunoreactive cells that localized predominantly in the distal gut. L-cells release GLP-2 along with GLP-1 in response to the chemical signals such as glucose (Roberge and Brubaker, 1993), fatty acids (Roberge and Brubaker, 1991) or a change of pH level in the intestinal lumen. Ileal GLP-2 immunoreactive cells are perfectly positioned to receive these chemical signals derived from the enteral nutrients. Most probably, GLP-2 is released in response to the intraluminal nutrients in the chicken intestine.

The present study showed that feed restriction had an impact on the frequency of occurrence of GLP-2-immunoreactive cells in the chicken small intestine. There were significant differences in the frequency of occurrence of GLP-2-immunoreactive cells among control and treated groups. This result revealed that larger number of L-cells containing GLP-2 in their cytoplasm are localized in the intestinal epithelium of restricted chickens than control ones. L-cells in the chicken small intestine are ‘open-type’ endocrine cells which have an apical cytoplasmic process accessing the intestinal lumen- like enteroendocrine cells containing other hormones (Hiramatsu *et al*., 2003, 2005; Nishimura *et al*., 2013). The ‘open-type’ enteroendocrine cells are considered as primary candidates for intestinal sensors (Breer *et al*., 2012). Several studies proved that ingested nutrients such as carbohydrates, proteins and fatty acids had considerable effects on L-cells in the mammalian intestine (Hansen *et al*., 2004; Cani *et al*., 2007; Karhunen *et al*., 2008; Yoder *et al*., 2009). Thus, ingested feed is the major signal that stimulates the GLP-2 (intestinal

peptide) release from L-cells as mentioned previously (Karhunen *et al*., 2008). In this study, the frequency of occurrence of GLP-2-immunoreactive cells is increased, especially in ileum of the chickens fed low amount of feed. L-cells in ileum from restricted chickens were located even in the upper part of villi than those from the control group. These results may indicate the retention of GLP-2 in L-cells due to the reduction in GLP-2 secretion from the small intestine. This was the consequence of the withholding of GLP-2 in L-cell cytoplasm by reason of decline in secretion stimulated by ingested feed. In previous experiment described in Chapter II I found almost the same trend in case of feed restriction in chicken on intestinal proglucagon-derived peptide (GLP-1). Thus these results suggest that the quantity of ingested feed had an influence on endocrine cells showing immunoreactivity for GLP-2 in the chicken small intestine. When feed intake of chickens declines or is restricted, villus height and crypt depth of small intestinal epithelium are found to decrease accordingly (Mitchell and Carlisle, 1992; Yamauchi *et al*., 1996; Hu and Guo, 2008), because stress of feed restriction delays the intestinal epithelial cell proliferation. Hu *et al*. (2010) reported that GLP-2 injection reversed the negative effect of stress on the weight and morphology and the absorptive function of small bowel of brioler chickens. Takahashi *et al*. (1992) reported that decreased synthesis of proglucagon-derived peptides by the intestine was largely responsible for the reductions in their circulating levels in starved rats. In this study, ileal GLP-2-immunoreactive cells were increased by the restricted feeding. The chickens were fed every day, and L-cells were stimulated by ingested diets accordingly. It is concluded that this difference in the result between Takahashi *et al*. (1992) and in the present study was depended on the difference in the feeding condition. The information gained from this study offers new insights into the regulatory mechanisms of GLP-2 in chickens.

Table 3. Comparison between chicken intestinal GLP-1 and GLP-2.

Parameters		Jejunum		Ileum	
		Proximal	**Distal**	**Proximal**	**Distal**
Cells/mm^2 (mean ± SD)	GLP-1*	2.33±1.33	17.32±5.56	24.89±7.25	29.93±8.9
	GLP-2	14.7±2.3	19.8±2.3	23.5±4.7	34.6±4.9
Shape of the cells	GLP-1 GLP-2	Almost same for the both, i. e., comma-like shape in crypts and spindle-like shape in villi.			
Localization of the cells	GLP-1	Abundantly both in crypts and villi area.			
	GLP-2	Mainly in the crypts and lower base of the villi area.			

* Data from Table 2.

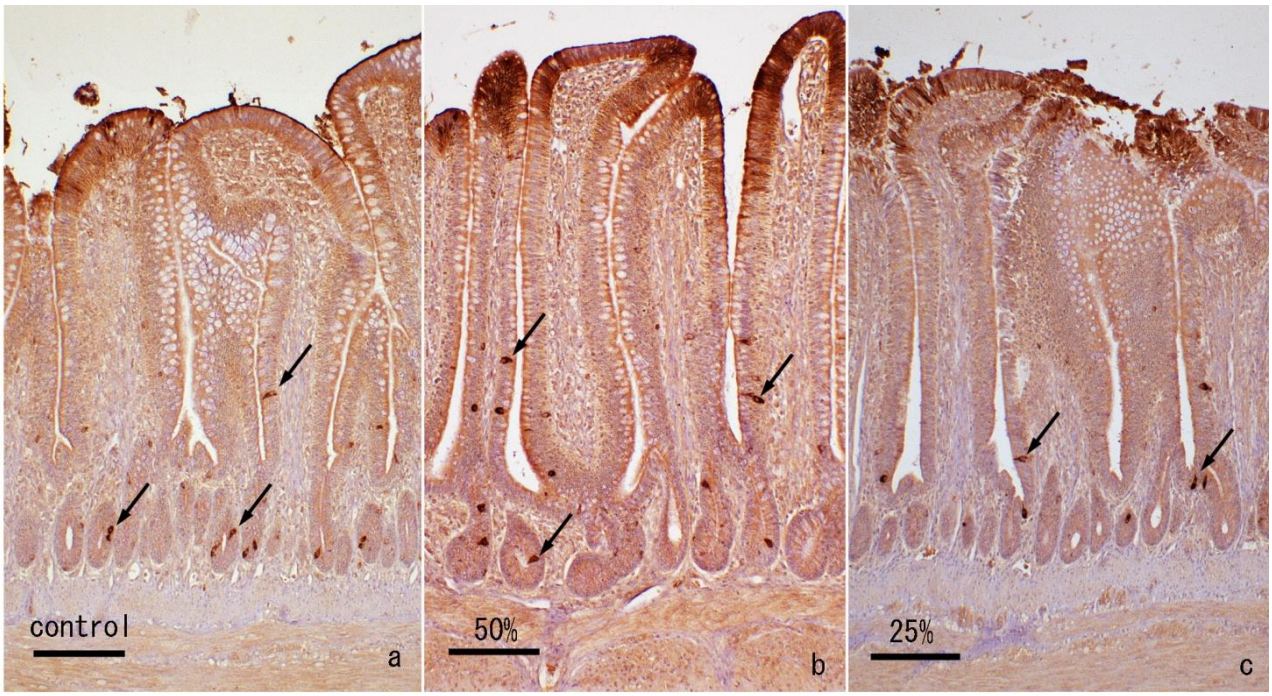

Fig. 6 GLP-2-immunoreactive cells (arrows) in the distal ileum from the control group (**a**), 50% feed supply group (**b**) and 25% feed supply group (**e**). Bar: 100 μm.

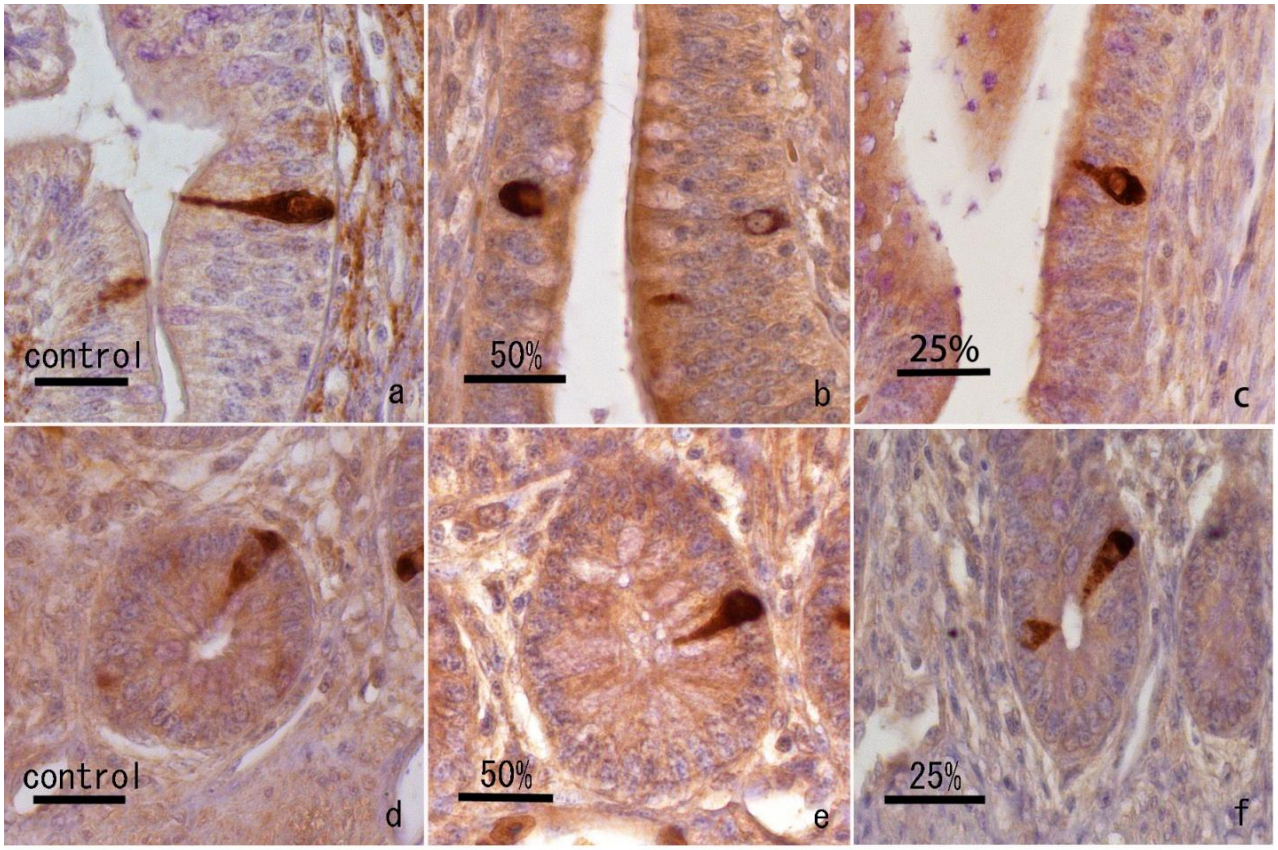

Fig. 7 High magnification views of GLP-2-immunoreactive cells in the distal ileum from the control group (**a, d**), 50% feed supply group (**b, e**) and 25% feed supply group (**c, f**). No obvious morphological differences are observed in villous epithelium (**a-c**) and crypts (**d-f**) among three groups. Bar: 20 μm.

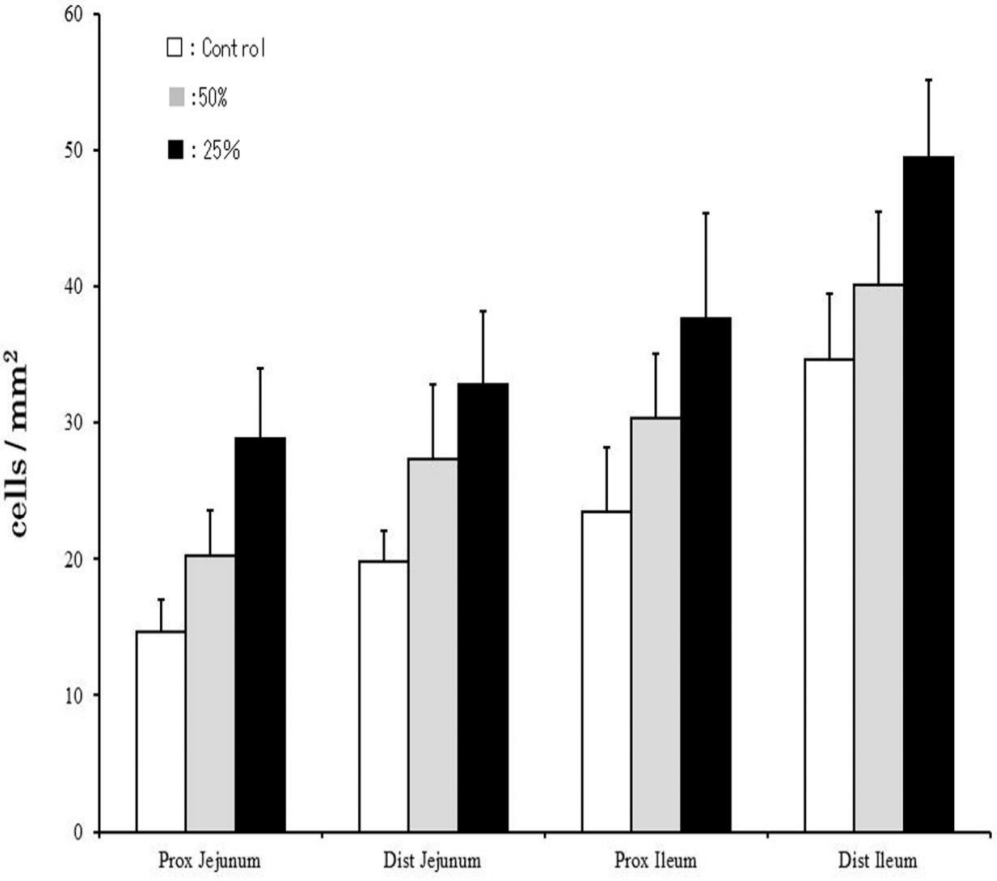

Fig. 8 Frequency of occurrence of GLP-2-immunoreactive cells in proximal and distal segment of jejunum and ileum from the control, 50% and 25% feed supply groups (cells/mm^2 mucosal area). There are significant differences among three groups in the same intestinal region and among four intestinal regions in the same group ($p<0.05$, error bars: SD).

III. Summary

The influence of restricted feeding on the distributional pattern of GLP-1 and GLP-2-immunoreactive endocrine cells in the chicken small intestine was investigated using immunohistochemical and morphometrical techniques. The present study demonstrated that the restricted feeding had an influence on the activity of both GLP-1- and GLP-2-immunoreactive cells in the chicken small intestine. The number of GLP-1- and GLP-2-immunoreactive cells were lowest in control group, medium in 50% and highest in 25% feed supply group at each intestinal region examined in this study, that is, increased significantly with the advancement of restricting the amount of feed supply ($p<0.05$). GLP-1- and GLP-2-immunoreactive cells in the control chickens were mainly located in epithelium from crypts to the lower part of intestinal villi. Those in restricted groups, however, tended to be located from crypts to the middle part of intestinal villi. These data shows that the quantity of food intake is one of signals which have an influence on the secretion of GLP-1 and GLP-2 from L-cells in the chicken small intestine.

Chapter IV

Influence of Dietary Protein Level on GLPs

IV. 1. Influence on GLP-1-immunoreactive cells in the chicken small intestine

IV. 1. 1. Introduction

GLP-1 is a potent incretin hormone produced in the intestinal L-cells by tissue-specific posttranslational processing of the proglucagon gene (Drucker, 2006; Richards and McMurtry, 2009). The well-characterized physiological effect of this meal-induced gut hormone (Elliott *et al*., 1993; Balkan, 2000; Feinle *et al*., 2003) is the rapid stimulation of insulin biosynthesis and release in a glucose-dependent manner. It also inhibits glucagon secretion, and increases pancreatic β-cell growth (Fridolf *et al*., 1991; Nauck *et al*., 1993; Farilla *et al*., 2002), reduces food intake (Furuse *et al*., 1997), decelerates gastric emptying (Nauck, 1998; Schirra *et al*., 2000) and intestinal motility (Tolessa *et al*., 1998a, b). In the chicken intestine, immunohistochemical and morphometrical studies have shown that the immunoreactive cells against GLP-1 antiserum are mainly distributed in the whole jejunum and ileum, and rarely found in ascending duodenum, but not in other intestinal regions (Hiramatsu *et al*., 2003; 2005). These findings suggest that GLP-1 plays a vital role in the regulation of chicken intestinal activities. GLP-1-secreting L-cells are scattered in the villous epithelium and crypts of the chicken small intestine. L-cells in the chicken small intestine are covered apically with microvilli, open-type, and contained many secretory granules in their perikarya (Nishimura *et al*., 2013). These ultrastructural features indicate that the secretion of GLP-1 is induced by the ingested feed in the chicken intestine. GLP-1 released into the circulation after a mixed meal (Kreymann *et al*., 1987) and dietary carbohydrate as well as fatty acids gives an account of this secretory action (Roberge and Brubaker, 1991; 1993). Judging from the role of GLP-1 in metabolism, dietary protein level might cause some alterations in its behavior. However, it is not yet clear whether dietary protein level has any influence on the secretion of GLP-1-immunoreactive cells in the

chicken small intestine. In the present study, it is aimed to clarify the influence of dietary protein level on GLP-1-immunoreactive endocrine cells in the chicken ileum using immunohistochemical and morphometrical methods. The significance of the protein ingestion on the secretion of GLP-1 from intestinal L-cells was also discussed in the present study.

IV. 1. 2. Materials and Methods

1. 2. 1. *Experimental birds*

White Leghorn day-old chicks (*Gallus gallus*) (n = 20, male) were commercially obtained from Komatsu Shukeijyo (Matsumoto, Nagano, Japan) and reared in our laboratory with standard procedures and identical management under continuous lighting up to 6 weeks of age.

1. 2. 2. *Experimental feeding*

After 3 days of adapting period, birds (average body weight 589.9 g) were randomly assigned into four dietary groups (CP 18%: control group, CP 9%, CP 4.5% and CP 0%) of five head each and fed in separate cages with the experimental diet shown in Table 4. The well-balanced experimental diets were isocaloric (ME = 2,850 Kcal/kg DM) with the same protein source. During the experimental feeding period of 7 days, daily feed intake (g/d) and body weight (g) of each bird were recorded every day at the same time (am 10:00) and by the same manner. Average value of the daily protein intake (g/d) of each chicken during the experimental period was calculated from average amount of the daily feed intake and CP inclusion level (%) of the experimental diet. Chickens were treated in accordance with the “Guideline for Regulation of Animal Experimentation (1997)” of Faculty of Agriculture, Shinshu University.

1. 2. 3. *Tissue samples*

At the end of the experimental feeding period, chickens were sacrificed by decapitation under anesthesia with diethyl ether. Distal ileum (at the middle level of ceca) about 2 cm

long was immediately dissected out from each bird after the gross anatomy of the abdomen. Contents of tissue samples were washed out with 0.75% sodium chloride solution and immersed in Bouin's fluid at room temperature for 24 h.

1. 2. 4. *Immunohistochemistry*

Tissue samples were embedded in paraffin wax according to standard procedures. Sections were cut at 5 μm thickness and used for the detection of GLP-1 by immunohistochemical technique. Streptavidin-biotin method (Guesdon *et al*., 1979) was applied to detect GLP-1-immunoreactive cells according to the procedures previously described by Hiramatsu and Ohshima (1995). Rabbit antiserum against synthetic GLP-1(1-19) conjugated to bovine serum albumin (Affiniti Research Products, UK, No.GA1176, diluted to 1:2000) was used as the primary antibody in this study. This antiserum shows no cross-reactivity with other proglucagon-derived peptides such as glucagon and GLP-2 (Tachibana *et al.,* 2005). Negative control sections were incubated with the normal rabbit serum instead of the specific primary antibody or in the absence of the primary antibody. They showed negative immunoreactivity.

1. 2. 5. *Morphometry*

The villous height (μm) of 10 well-oriented villi was measured from each chicken. Fifty villi in total were measured per group and the average value was calculated for each group. To evaluate the frequency of occurrence of GLP-1-immunoreactive cells in each group, the immunoreactive cells with clearly identifiable nuclei were counted, and the area of the mucosal layer was measured. The cell number per area of the mucosal layer (cells/mm^2) was then calculated. These measurement and quantification were carried out by 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 5 chickens in each group.

1. 2. 6. *Statistical analysis*

Statistical analysis was performed to assess the differences in the villous height and the frequency of GLP-1-immunoreactive cells among four groups using Tukey's method (Yanai, 2011). Multiple regression analysis using the 'General Linear Model Procedures' in SAS/STAT (SAS Institute Inc., Cary, NC, USA) was performed between the daily protein intake and the frequency of occurrence of GLP-1-immunoreactive cells.

IV. 1. 3. Results

1. 3. 1. *Influences of dietary protein level on body weight gain, feed intake and villous height and histology*

Weight gains were declined correspondingly with diminishing of dietary protein level and negatively balanced in CP 0% group. There was, however, no significant difference in the daily feed intake among four groups (Table 5). Villous height of CP 0% and CP 4.5% groups tended to be lower than that of the control group but there were no significant differences. The villous tips tended to be dull in CP 0% and CP 4.5% groups comparing with those of the CP 18%: control group (Fig. 9).

1. 3. 2. *Influences of dietary protein level on GLP-1-immunoreactive cells*

Large number of endocrine cells exhibited immunoreactivity for GLP-1 antiserum and they were observed scattering in the ileal epithelium of all groups (Fig. 9a-d). Most of the cells had pyramidal or spindle-like shape in the villous epithelium (Fig. 10a, c) and comma-like shape in crypts (Fig. 10b, d) and were in contact with the intestinal lumen through their apical cytoplasmic process. GLP-1-immunoreactive cells showing round or oval in shape were observed in lower CP (4.5% and 0%) groups and their number turned to be increased in CP 0% group (Fig. 10c, arrows). In the control group, GLP-1-immunoreactive cells were primarily dispersed in crypts and epithelium of lower parts of intestinal villi (Fig. 9a). In CP

0% and CP 4.5% groups, however, these cells were likely to be more noticed in crypts and to be fewer in epithelium of the middle part of intestinal villi (Fig. 9c, d).

In the control group, the frequency of occurrence of GLP-1-immunoreactive cells was 41.1 ± 4.1 (cell number per mucosal area: cells/mm^2, mean ± SD). On the other hand, in case of CP 9%, CP 4.5% and CP 0% groups these values were 38.5 ± 4, 34.8 ± 3.1 and 34.3 ± 3.7, respectively. There were significant differences in the frequency of occurrence of GLP-1-immunoreactive cells between the control group and lower CP level (4.5% and 0%) groups ($p<0.05$). The frequencies of occurrence of GLP-1-immunoreactive cells were decreased with the steady decline of the dietary CP level (Fig. 11). The daily protein intake (average of the daily feed intake × CP% / 100) were calculated for each bird in each group (Table 5). Multiple regression analysis using the 'General Linear Model Procedures' indicated a significant correlation between the daily protein intake (X) and the frequency of occurrence of GLP-1-immunoreactive cells (Y). The regression equation was as follow: $Y = 31.65 + 1.13X$, $p<0.0001$ (Fig. 12).

IV. 1. 4. Discussion

The secretion of GLP-1 from intestinal L-cells is known to be stimulated in response to ingestion of mixed meals or macronutrients in mammals (Elliott *et al.,* 1993; Balkan, 2000). However, it has not yet been clear that a specific nutrient could stimulate the GLP-1 secretion from L-cells in the chicken intestine. The present study demonstrated that the protein ingestion had an impact on the secretion of GLP-1 from L cells in the chicken ileum. Frequencies of occurrence of GLP-1-immunoreactive cells were decreased with the steady decline of the dietary CP level and significant differences were recognized between the control group and lower CP level (4.5% and 0%) groups. The positive correlation was also ascertained between the frequency of occurrence of GLP-1-immunoreactive cells and the daily protein intake. These results indicate that the amount of ingested protein or its metabolite is one of important triggers which stimulate GLP-1 secretion from L-cells in the

chicken small intestine. L-cells are open-typed cells in contact with intestinal lumen by their cytoplasmic processes covered with microvilli and play as the chemosensor monitoring nutrients in the ingested food (Breer *et al*., 2012). Recently it is demonstrated that L-cells in the chicken small intestine were covered with microvilli apically (Nishimura *et al*., 2013) and in Chapter III it is revealed that the restricted feeding influenced on their frequency of occurrence. Thus, L-cells might monitor the content of ingested feed and secrete GLP-1 to conduct physiological phenomena in the chicken. The present study demonstrated that quantity of ingested protein was one of important factors which could influence GLP-1 secretion.

Several studies have shown that ingestion of specific nutrients such as carbohydrates, proteins and fatty acids affect on GLP-1 secretion from intestinal L-cells in mammals including human (Hansen *et al*., 2004; Cani *et al*., 2007; Karhunen *et al*., 2008; Yoder *et al*., 2009). Carbohydrate is one of strong stimuli of GLP-1 release from intestinal L-cells (Elliott *et al.*, 1993; Herrmann *et al*., 1995). Some studies indicated that the glucose ingestion influenced GLP-1 secretion in healthy humans (Qualmann *et al*., 1995; Salehi *et al.*, 2008). Herrmann *et al*. (1995) demonstrated that the oral administration of glucose induced GLP-1 release, but intravenous glucose did not. Yoder *et al*. (2010) showed the dose-dependent relationship between GLP-1 secretion and amounts of dietary carbohydrate in lean rats. Fat also induces GLP-1 secretion. Yoder *et al.* (2009) showed that lipid stimulated GLP-1 secretion in a dose-dependent manner. But the increase of GLP-1 secretion by fat ingestion is delayed comparing with carbohydrates (Elliot *et al*., 1993). Thus, carbohydrate and fat are stimuli to GLP-1 secretion from L-cells in the mammalian intestine.

Several studies showed that protein ingestion could stimulate GLP-1 release from intestinal L-cells. GLP-1 outputs, however, did not respond dose-dependently to increasing amounts of dietary protein in lean rats (Yoder *et al*., 2010). Elliott *et al*. (1993) and

D'Alessio *et al*. (1993) noted protein as a potent stimulus for GLP-1 secretion in healthy humans. Effects of ingested protein on GLP-1 secretion differ according to their sources because of their different amino acid profile.

Present findings, as mentioned above, indicate that GLP-1 activity is respondent to protein ingestion in chickens. This response in chicken may be dependent on amounts of protein intake, because the frequency of occurrence of GLP-1-immunoreactive cells was significantly decreased in a parallel manner with the reduction of the daily protein intake. GLP-1-immunoreactive cells in lower CP level groups, moreover, showed different localization and morphology compared with those in the control group. These data lead to a hypothesis that ingested protein regulates not only GLP-1 secretion but also proliferation of L-cells in chicken ileum. A possible mechanism associated with this effect was the stimulation of intestinal L-cells by quantities of dietary amino acids present in CP or alternatively, interactive calori-protein ratio of the ration supplied. Yusta *et al.* (2000) showed that L-cells in the human small intestine co-express GLP-2-receptor with GLP-1 immunoreactivity. GLP-2 is also released from L-cells in response to ingestion of meals and promotes crypt cell proliferation. In the chicken small intestine, GLP-1 and GLP-2 are co-stored in the same secretory granules of L-cells (Nishimura *et al*., 2013). Reduction of the daily protein intake induces a decreased secretion of GLP-1 and GLP-2 as proliferation of L-cells might be suppressed and the frequency of occurrence of GLP-1-immunoreactive cells was cut downed. Till now there are no documented evidence showing an expression of GLP-2 receptor in L-cells of the chicken small intestine. Systematic biochemical studies are necessary to resolve this crucial issue.

The mechanism of GLP-1 secretion is very complex because many circulating or locally derived peptide-hormones are concerned in it. Glucose-dependent insulinotropic polypeptide (GIP) is one of such hormones. This hormone is secreted from K-cells in response to the feed ingestion and plays as the incretin hormone. In the mammalian

intestine, K- and L-cells are localized in the proximal and distal small intestine, respectively (Buchan *et al*., 1978; Bunnett and Harrison, 1986; Damholt *et al.*, 1999). It is said that the inhibition of intestinal motility by GLP-1 comes out through an indirect effect via central and enteric nervous system (Tolessa *et al*., 1998a, b; Stanley *et al*., 2004). In human, GLP-1 exhibits a biphasic profile with early and late phases following meal ingestion (Herrmann *et al*., 1995). Because GIP stimulates GLP-1 secretion from L-cells in mammals (Roberge and Brubaker, 1993; Damholt *et al.*, 1998), early phase of GLP-1 secretion is induced by GIP and late phase due to nutrients in the intestinal lumen. Recently, GIP-immunoreactive (K) cells were reported in villous epithelium and crypts of chicken small intestine (Pirone *et al.*, 2011). GIP shows a high response after protein ingestion in healthy men (Carr *et al*., 2008). It is likely that GLP-1 secretion is regulated by GIP in the chicken small intestine.

The present data are relevant to the conclusion that the protein ingestion makes an impact on L-cells in the chicken small intestine since the frequencies of occurrence of GLP-1-immunoreactive cells were decreased with the steady decline of the dietary CP level and showed a significant correlation with the amount of daily protein intake. We evaluated the effects of dietary protein levels on GLP-1 containing cells in the small intestine of young chicken. Long-term studies would be of interest in adult chicken to elucidate the effect of protein supplementation on this mechanism. An alternative approach, e. g., the feeding of protein from various sources, could also be evaluated to determine whether the stimulation of GLP-1 could how much affected or not.

Table 4. Composition of experimental diets used in this study (g). They were isocaloric (ME=2,850 Kcal/kg DM) with the same protein source (ISP = Isolated Soybean Protein).

Composition	**% CP**			
	18	**9**	**4.5**	**0**
ISP (CP 84%)	214.0	107.0	53.5	0
L-Methionine	1.3	0.7	0.4	0.0
L-Cystine	2.2	1.1	0.6	0.0
Cornstarch	493.1	599.1	652.1	705.2
Cellulose	196.4	199.1	200.4	201.8
Corn oil	30	30	30	30
Mineral mixture	58.5	58.5	58.5	58.5
Vitamin mixture	2.0	2.0	2.0	2.0
Choline chloride	1.5	1.5	1.5	1.5
Inositol	1.0	1.0	1.0	1.0
Total	1000	1000	1000	1000

Table 5. Body weight gain (g), daily feed and protein intake (g/head/day) and villous height (μm) of chickens from 4 groups (CP 18%: control, 9%, 4.5% and 0%). Values are group means ± SD. There are significant differences in body weight gain between different alphabets (n = 5, $p<0.01$). There are no significant differences in daily feed intake and villous height.

Parameters	**Dietary CP level (%)**			
	18	**9**	**4.5**	**0**
Body weight gain (g)	96.4 ± 16.1^{a}	78.4 ± 34.7^{a}	28.8 ± 17.6^{b}	-41.4 ± 11.1^{c}
Daily feed intake (g/head/day)	51.4 ± 7.3	57.0 ± 11.5	53.2 ± 9.6	46.9 ± 5.5
Daily protein intake (g/head/day)	9.3 ± 1.1^{a}	5.1 ± 0.9^{b}	2.4 ± 0.4^{c}	0 ± 0^{d}
Villous height (μm)	396.6 ± 72.5	399.3 ± 68.9	379.1 ± 73.8	384.5 ± 66.1

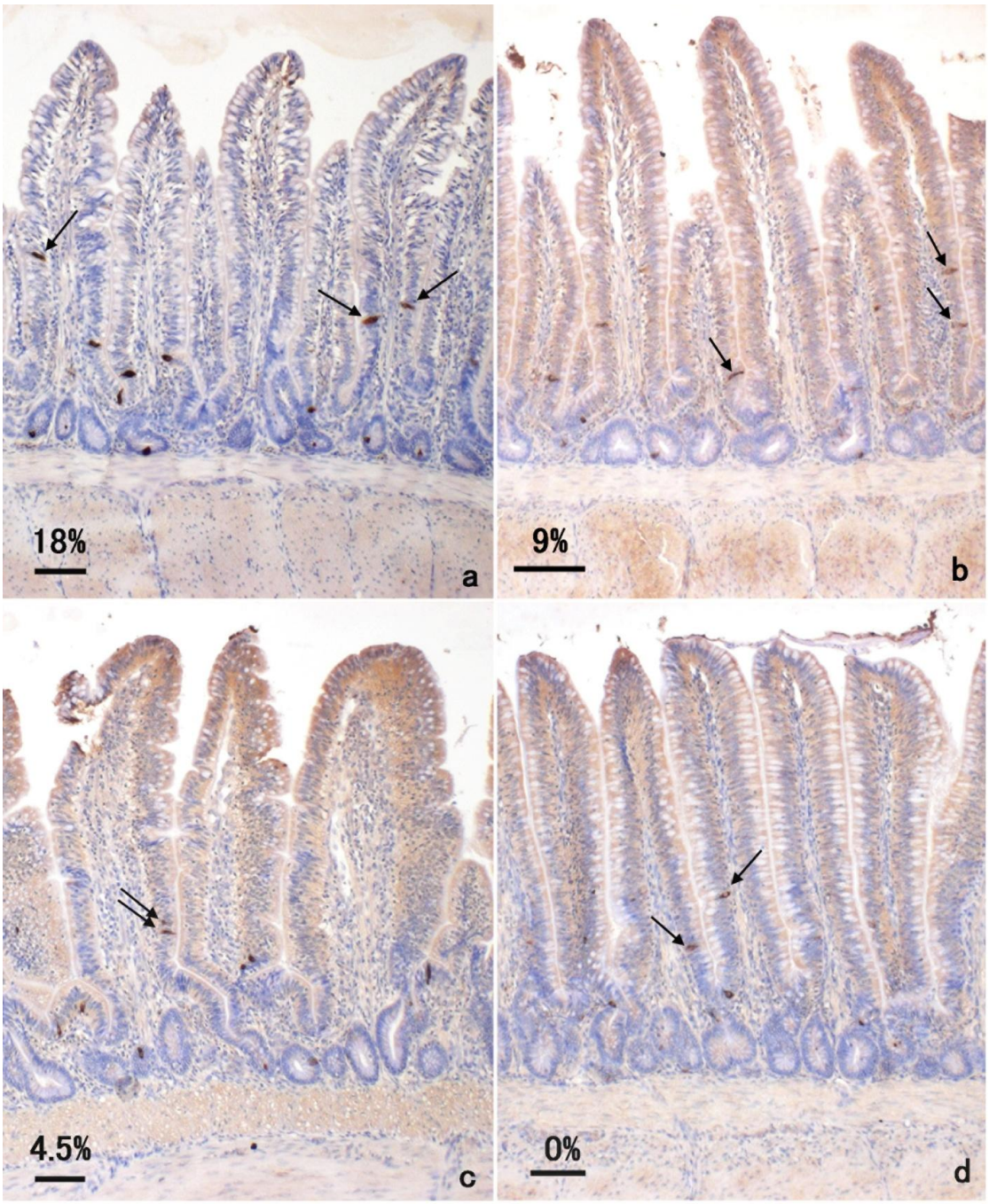

Fig. 9 GLP-1-immunoreactive cells (arrows) in the distal ileum from CP 18% (control, **a**), 9% (**b**), 4.5% (**c**) and 0% (**d**) groups. Bar: 100 μm.

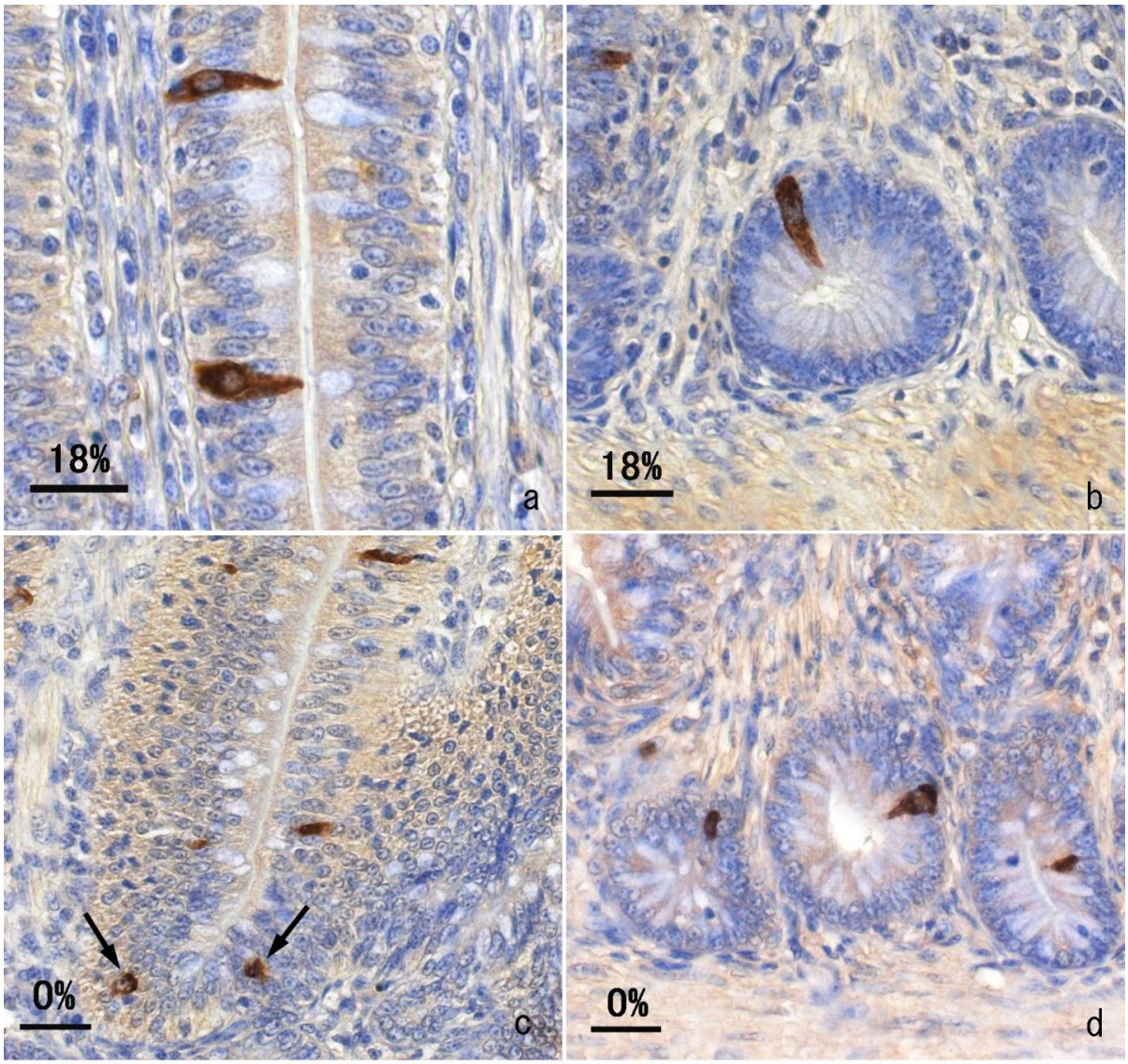

Fig. 10 High magnification views of GLP-1-immunoreactive cells in the distal ileum from CP 18% (control) group (**a, b**) and CP 0% group (**c, d**). No obvious morphological differences are observed in crypts (**b, d**), but GLP-1-immunoreactive cells in a round shape are increased in the villous epithelium of CP 0% group (**c,** arrows) Bar: 20 μm.

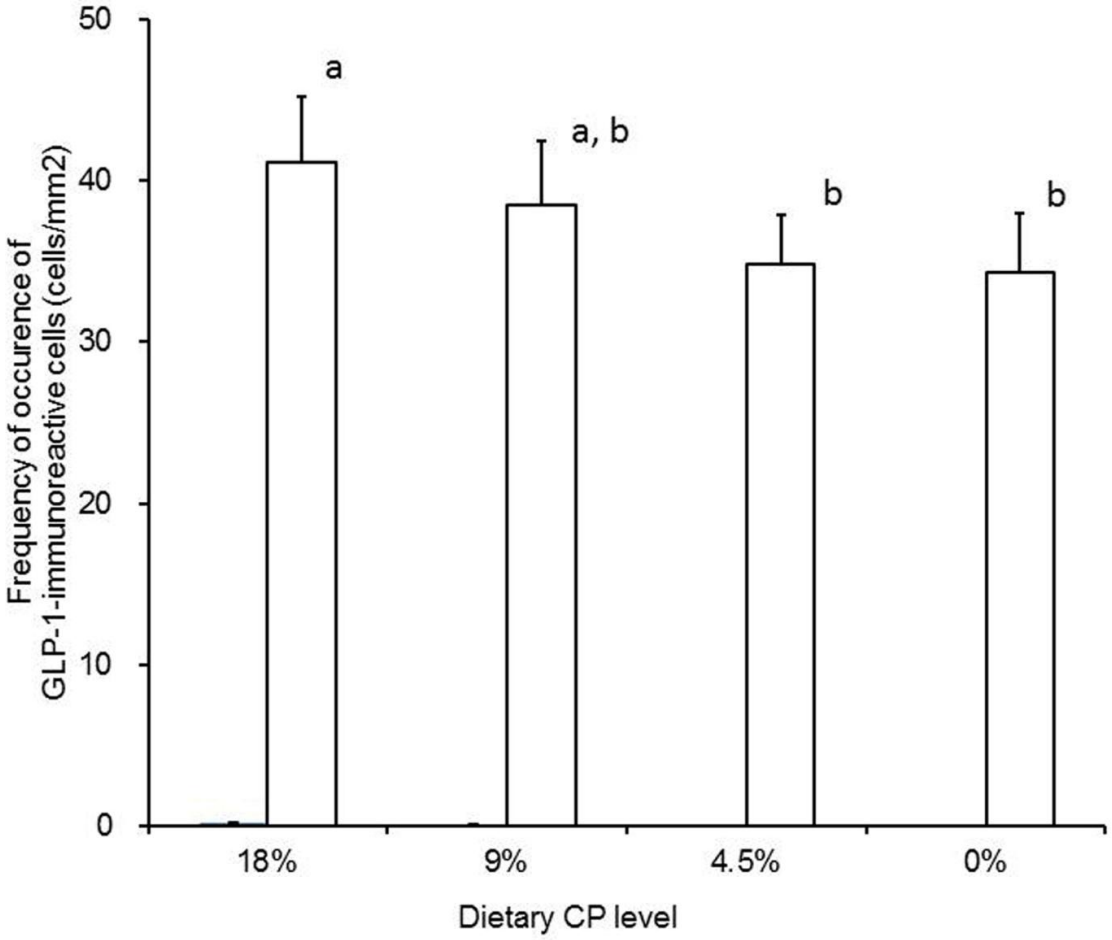

Fig. 11 Frequency of occurrence of GLP-1-immunoreactive cells in the distal ileum from CP 18% (control), CP 9%, CP 4.5% and CP 0% groups (cells/mm^2 mucosal area). There are significant differences between different alphabets ($p<0.05$, error bars: SD).

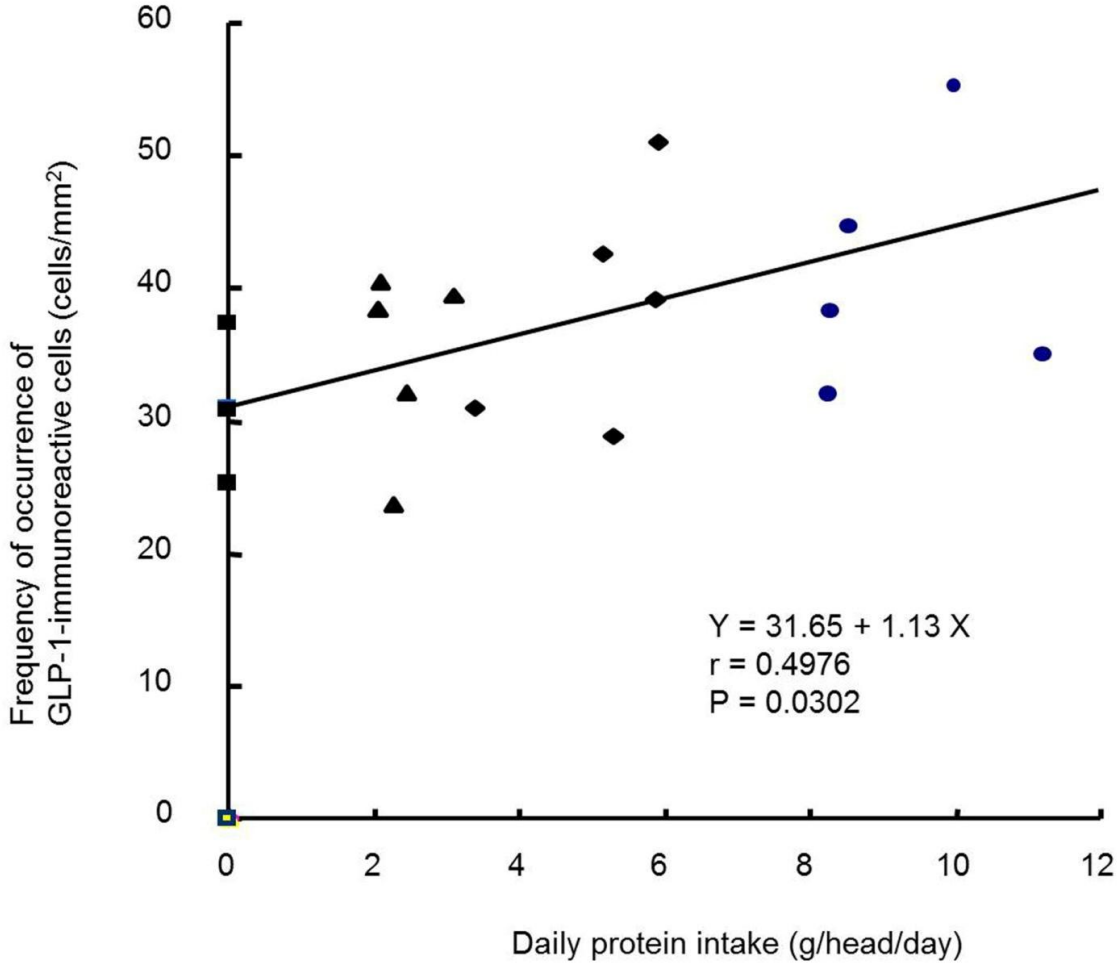

Fig. 12 Regression line of the frequency of occurrence of GLP-1-immunoreactive cells (Y) on the daily protein intake (X). The equation of this line is Y = 31.65 + 1.13X, $p<0.0001$.

IV. 2. Influence on GLP-2-immunoreactive cells in the chicken small intestine

IV. 2. 1. Introduction

GLP-2 plays important roles in the maintenance of energy homeostasis through acute and chronic effects on gut motility, and nutrient ingestion, digestion, absorption, and storage, as well as mobilization (Kieffer and Habener 1999; Drucker, 2001b, 2002; L'Heureux and Brubaker 2001; MacDonald *et al*., 2002; Holst, 2002; Baggio and Drucker, 2004). GLP-2 is synthesized and secreted by enteroendocrine cells localized in the intestinal epithelium (Eissele *et al*., 1992). GLP-2 promotes the growth and survival of epithelial cells within the intestinal mucosa via an inhibition of apoptotic cell death and a stimulation of cellular proliferation (Drucker, 2002). GLP-2 also enhances barrier function and increases the resistance to and recovery from a variety of experimental models of gut injury (Drucker, 2002). Recent studies have established that the regulation of GLP-2 secretion is highly complex, involving direct effects of ingested nutrients, as well as indirect effects through neural and endocrine pathways. The focus of this experiment is on regulatory mechanisms of the dietary protein level influencing the secretion of GLP-2 from the chicken small intestine.

IV. 2. 2. Materials and Methods

2. 2. 1. *Experimental birds*

White Leghorn day-old chicks (*Gallus gallus*) (n = 20, male) were commercially obtained from Komatsu Shukeijyo (Matsumoto, Nagano, Japan) and reared in our laboratory with standard procedures and identical management under controlled light condition (12 h light: 12 h darkness) up to 6 weeks of age.

2. 2. 2. *Experimental feeding*

After 3 days of adapting period, birds (average body weight 589.9 g) were randomly assigned into four dietary groups (CP 18%: control group, CP 9%, CP 4.5% and CP 0%) of

five head each and fed in separate cages with the experimental diet shown in Table 4. The well-balanced experimental diets were isocaloric (ME = 2,850 Kcal/kg DM) with the same protein source. During the experimental feeding period of 7 days, daily feed intake (g/d) and body weight (g) of each bird were recorded every day at the same time (am 10:00) and by the same manner. Average value of the daily protein intake (g/d) of each chicken during the experimental period was calculated from average amount of the daily feed intake and CP inclusion level (%) of the experimental diet. Chickens were treated in accordance with the "Guideline for Regulation of Animal Experimentation (1997)" of Faculty of Agriculture, Shinshu University.

2. 2. 3. *Tissue samples*

After experimental period, all the birds were sacrificed by decapitation under anesthesia. Intestinal tissue samples about 2cm long were rapidly dissected out from each bird, washed with 0.75% NaCl solution and immersed in 4% paraformaldehyde (PFA) for 24 hours at 4˚C. Samples were collected from descending and ascending duodenum, proximal and distal jejunum and ileum, colorectum and proximal and distal regions of cecum.

2. 2. 4. *Immunohistochemistry*

The PFA-fixed tissue samples were embedded in paraffin wax according to the standard procedures. Sections were cut at 5μm thickness using sliding microtome, rehydrated in graded ethanol followed by xylene solution. To retrieve the hind epitope caused by PFA-based methylene bridges, 0.5% antigen retrieval medium (citraconic anhydride, Immunosaver®, Nisshin EM Corporation, Tokyo, Japan) was applied for 45 min at 98˚C. The incubated sections were then washed three times (five min each) with phosphate buffered saline (PBS). Rabbit antiserum against synthetic GLP-$2_{(1\text{-}34)}$ conjugated to bovine serum albumin (Phoenix Pharmaceuticals Inc. USA, H-028-14, diluted to 1:2000) was used as the primary antibody at room temperature overnight in a moisture box. The HRP-labeled

streptavidin-biotin method (Guesdon *et al.*, 1979) was applied to detect GLP-2 immunoreactive cells according to the procedure previously described by Hiramatsu and Ohshima (1995). After counterstaining with Mayer's hematoxylin, sections are mounted, coverslipped and observed under light microscope. The specificity of primary antibody used in this study against GLP-2 was documented by Nishimura *et al*. (2013).

2. 2. 5. *Morphometry*

To evaluate the frequency of occurrence of GLP-2-immunoreactive cells in each intestinal region, we only counted the cells with clearly identifiable nuclei, and the area of the mucosal layer was also measured accordingly. The number of cells per area of the mucosal layer (cells/mm^2) was then calculated. This quantification was carried out using computerized image analyzing system (KS400, ZEISS, Göttingen, Germany). Twenty areas were measured in each intestinal region from each bird, i. e, 400 areas in total from 5 chickens of each group.

2. 2. 6. *Statistical analysis*

Tukey's method (Yanai, 2011) of statistical analyses were performed to assess the differences in the frequency of occurrence of GLP-2 immunoreactive cells among the four dietary groups. P values less than 0.05 were considered as statistically significant.

IV. 2. 3. Results

2. 3. 1. *Influences of dietary protein level on GLP-2-immunoreactive cells*

Endocrine cells showing immunoreactivity for GLP-2 antiserum were present with sparse order only in the whole jejunum and ileum but not in the other intestinal regions of four groups. In the control group, GLP-2-immunoreactive cells were largely distributed in crypts and epithelium of middle and lower parts of intestinal villi (Fig. 13a). In other three groups, however, fewer number of immunoreactive cells were present in epithelium of the middle part of intestinal villi (Fig. 13b). Most of the cells had pyramidal or spindle-like shape in the villi area (Fig. 14a, c) and comma-like shape in crypts (Fig. 14b, d) and were in

contact with the intestinal lumen through their slender apical cytoplasmic process. Round or oval shaped GLP-2-immunoreactive cells were found in lower CP (0% and 4.5%) groups and their number turned to be increased in CP 0% group (Fig. 14c, arrow).

2. 3. 2. *Morphometry*

Table 6 indicates the frequency of occurrence of GLP-2 immunoreactive cells in different portion of intestinal ileum and jejunum. More number of cells were distributed in the distal than the proximal region. There were significant differences in the frequency of occurrence of GLP-2-immunoreactive cells between control and the treatment groups as well as within the treatment groups ($p<0.05$). The frequencies of occurrence of GLP-2-immunoreactive cells were decreased with the advancement of restricting the amount of dietary % CP supply (Fig. 15). There were no significant differences in villous heights among the birds.

IV. 2. 4. Discussion

GLP-2 is secreted from intestinal L-cells, the majority of which are located in the distal ileum (Sjölund *et al*., 1983). The major stimulus for GLP-2 secretion is the size and nutrient composition of the ingested meal (Xiao *et al*., 1999; Vilsbøll *et al*., 2001). GLP-2 secretion depends on the specific nutrient composition of the meal, and there are clear differences in the response to proteins, carbohydrates, and lipids (Dubé and Brubaker, 2004). The results of this experiment agree with the findings of Dumoulin *et al*. (1998) who stated that peptones or protein hydrolysates are stimulatory on GLP secretion in rat ileum. Reimer *et al*. (2001) also demonstrated the same fact in case of the NCI-H716 human cell line. This may be due to drastic changes in calorie-protein ratio in the diet supplied. Calorie-protein ratio in the diet is a crucial factor to maintain the normal physiological homeostasis in monogastric animal. Ingested mixed meal carbohydrates are potent GLP secretagogues *in vivo*, and circulating GLP-2 level increased (from fasting level of 15-20 pmol/l) by two-to-threefold in humans following glucose ingestion (Xiao *et al*., 1999). In agreement with the

morphological appearance of the L-cells, several studies on secretion of proglucagon derived peptides (PGDPs), have indicated that it is the physical contact between luminal nutrients and the microvilli of the L-cells that appears to stimulate their secretion of GLP-2 (Ørskov and Holst, 1987; Hoyt *et al*., 1996; Brubaker *et al*., 1997a; Jeppensen *et al*., 1999; Rubin, 1999; Xiao *et al*., 1999; Burrin *et al*., 2000; Hartmann *et al*., 2000). Several of the nutrients, which have been shown to be more potent in enhancing gut structure and function, seem to affect the release of PGDPs. This phenomenon has been investigated in various experimental models. The majority of these studies have been on GLP-1 secretion, but since both GLP-1 and GLP-2 are cleaved from proglucagon and released synchronously from the L-cells, the results obtained for GLP-1 can be extrapolated to include GLP-2 (Thulesen, 2004). Partial hydrolysates of proteins (peptones), which mimic the protein-derived components of chyme, have been shown to be strong secretagogues of the intestinal release of PGDPs in rat ileum (Cordier-Bussat *et al*., 1998). The findings on GLP-2 secretion in response to luminal exposure of L-cells to nutrients are consistent with the hypothesis that GLP-2 physiologically may act to ensure optimal intestinal capacity (by simulation of intestinal growth and functional capacity). Thus in conditions with proximal intestinal insufficiency, relatively large amounts of nutrients or partially processed nutrients may reach the distal region of the small intestine, and thereby make physical contact with the L-cells possible, stimulating the release of GLP-2, which in turn, induces adaptive proliferation (Thulesen, 2004). In conclusion, it is suggested that dietary protein level had effects on GLP-2 secretion from chicken intestinal L-cells. Further systematic studies are required to understand how the type and amount of amino acids specially limiting amino acids (lysine and methionine) influence in the secretory mechanisms of GLP-2 in chicken intestine.

Table 6. Frequency of occurrence of GLP-2-immunoreactive cells in different portion of intestinal ileum and jejunum of chicken of 4 (CP 18%: control, 9%, 4.5% and 0%) groups. Values represent mean number of GLP-2-immunoreactive cells per 1 mm^2 mucosa. There are significant differences between different alphabets ($p<0.05$, error bars: SD).

Intestinal segments	**% CP**			
	18	**9**	**4.5**	**0**
Proximal Jejunum	20.12 ± 4.29^{a}	16.49 ± 3.27^{a}	14.62 ± 4.65^{a}	12.77 ± 5.19^{a}
Distal Jejunum	23.83 ± 2.46^{b}	19.67 ± 1.85^{b}	16.39 ± 2.09^{b}	14.52 ± 2.06^{b}
Proximal Ileum	25.63 ± 2.08^{c}	21.78 ± 2.36^{c}	19.54 ± 1.32^{c}	16.29 ± 1.32^{c}
Distal Ileum	29.08 ± 0.58^{d}	24.42 ± 1.59^{d}	21.75 ± 1.91^{d}	17.74 ± 2.56^{d}
Average	24.67 ± 2.35^{a}	20.59 ± 2.27^{b}	18.08 ± 2.48^{c}	15.33 ± 2.78^{d}

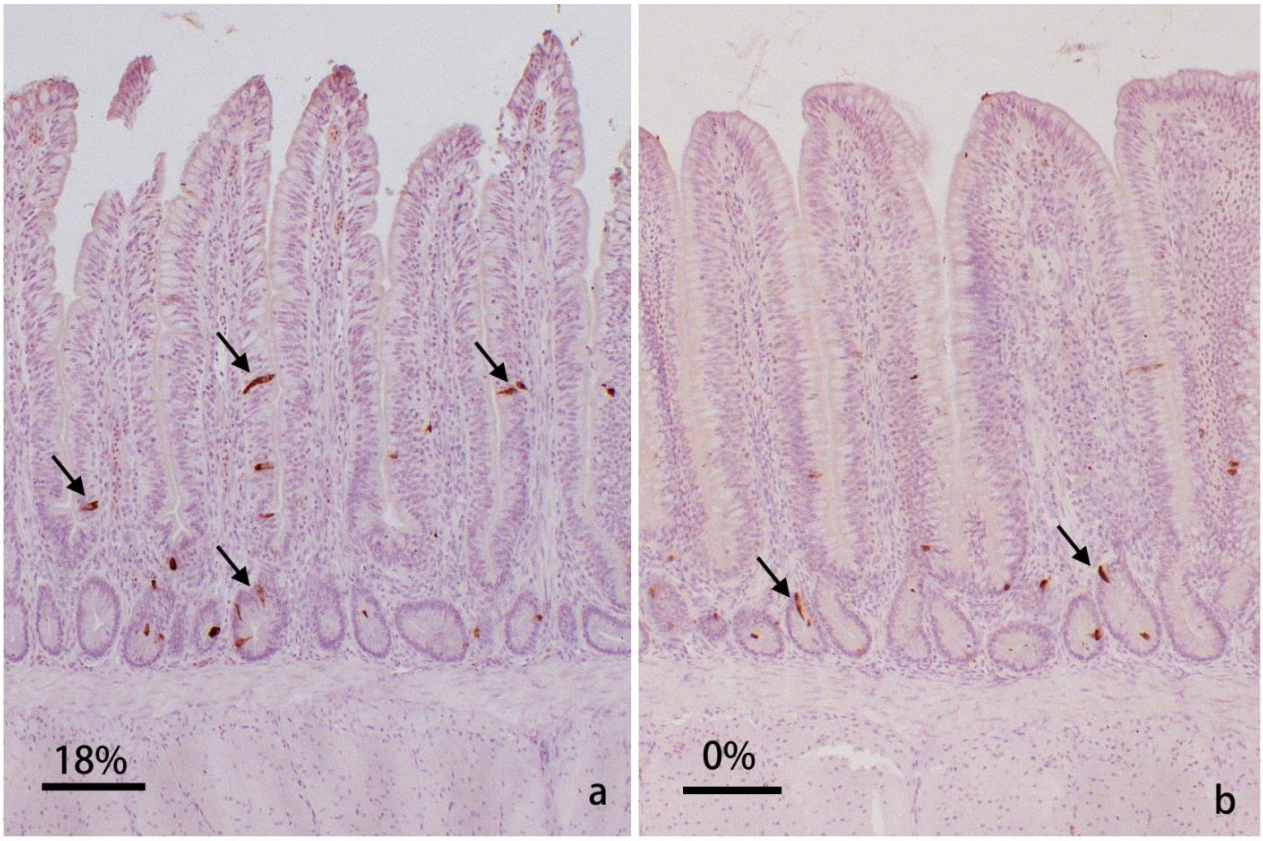

Fig. 13 GLP-2-immunoreactive cells in the distal ileum from CP 18% (control, **a**) and 0% (**b**) groups. Arrows indicate GLP-2-immunoreactive cells. Bar: 100 μm.

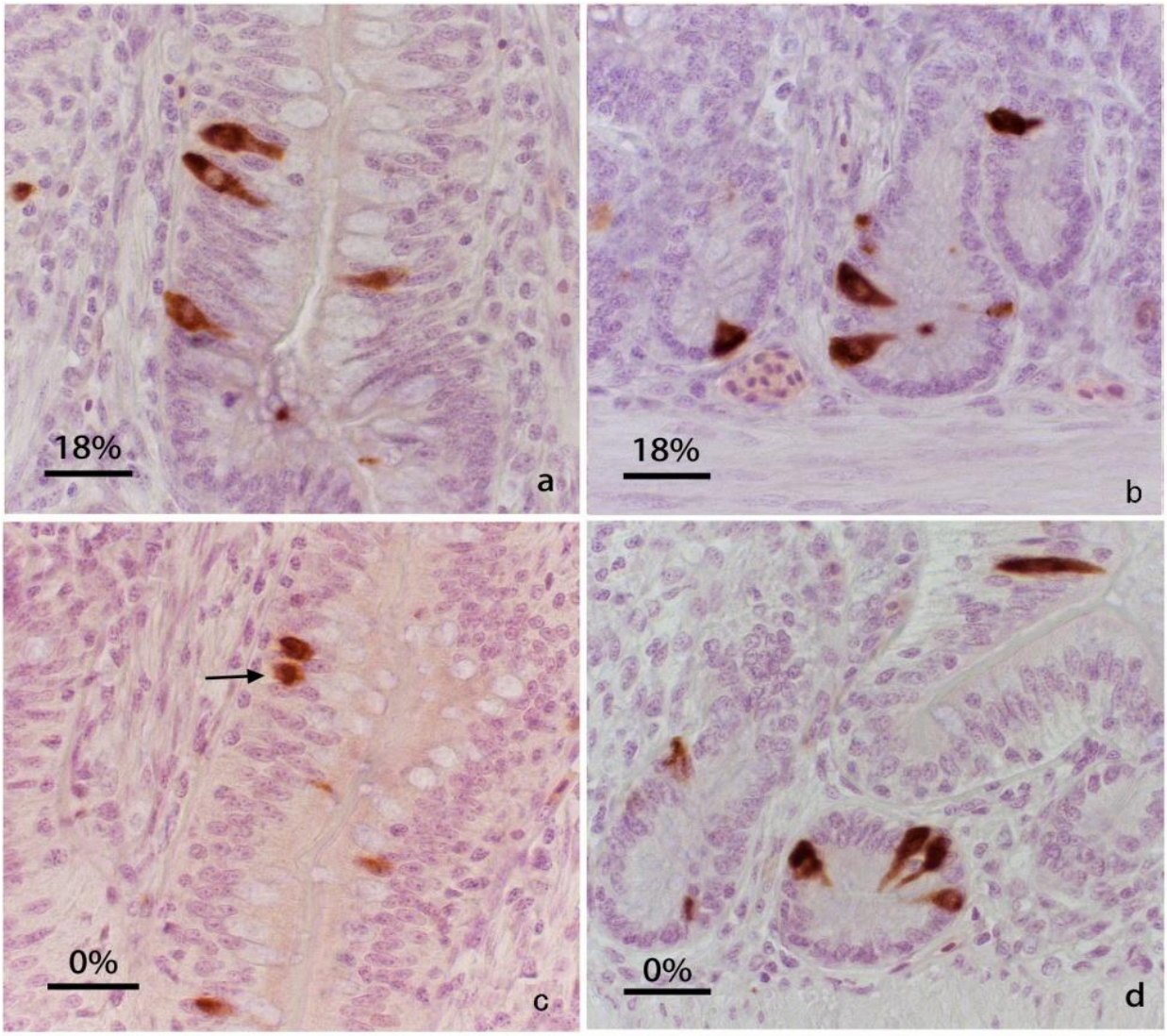

Fig. 14 GLP-2-immunoreactive cells in the distal ileum from CP 18% (control, **a**, b) and 0% (c, **d**) groups. **a, b:** High magnification views of GLP-2-immunoreactive cells in the villous epithelium of the distal ileum. Arrow in **b** shows GLP-2-immunoreactive cell in an oval shape. Bar: 20 μm. **c, d:** High magnification views of GLP-2-immunoreactive cells in crypts of the distal ileum. No obvious morphological differences are observed. Bar: 20 μm.

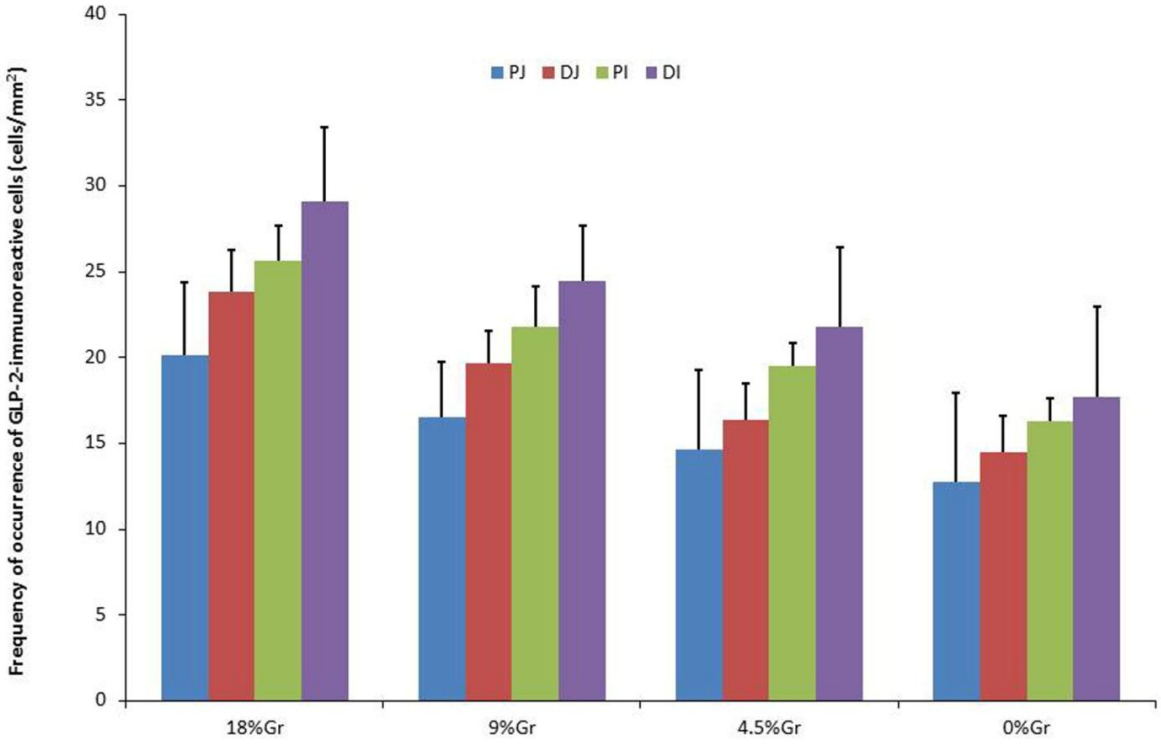

Fig. 15 Frequency of occurrence of GLP-2-immunoreactive cells in proximal and distal segment of jejunum and ileum from CP 18% (control), CP 9%, CP 4.5% and CP 0% groups (cells/mm^2 mucosal area). There are significant differences among four groups in the same intestinal region and among four intestinal regions in the same group ($p<0.05$, error bars: SD).

IV. Summary

The significance of dietary protein level on GLP-1- and -2-immunoreactive cells in the chicken intestine were investigated using immunohistochemical and morphometrical techniques. The present study showed that the protein ingestion had an impact on the activities of both GLP-1 and -2-immunoreactive cells. Weight gains were declined correspondingly with diminishing of dietary protein level, but no significant differences were detected in the daily feed intake and villous height. Frequencies of occurrence of GLP-1-immunoreactive cells were 41.1 ± 4.1, 38.5 ± 4, 34.8 ± 3.1 and 34.3 ± 3.7 (cell numbers per mucosal area: cells/mm^2, mean ± SD) for dietary crude protein (CP) level of 18% (control), 9%, 4.5% and 0 % groups, respectively and significant differences were recognized between the control and lower CP (4.5% and 0%) groups ($p<0.05$). Multiple regression analysis indicated a significant correlation between the daily protein intake and the frequency of occurrence of GLP-1-immunoreactive cells. Plasma concentration of GLP-1 showed no significant differences among four groups. On the other hand, the frequencies of occurrence of GLP-2-immunoreactive cells were 24.67 ± 2.35, 20.59 ± 2.27, 18.08 ± 2.48 and 15.33 ± 2.78 (cell numbers per mucosal area: cells/mm^2, mean ± SD) for dietary CP level of 18% (control), 9%, 4.5% and 0 % groups, respectively and there were significant differences between the control and CP treatment groups ($p<0.05$). These data shows that the protein ingestion is one of the signals which have an influence on the secretion of GLP-1 and GLP-2 from chicken small intestine.

Chapter V

General Conclusion

General Conclusion

The intestinal proglucagon-derived glucagon-like peptides, GLP-1 and GLP-2, are key regulators of nutrient homeostasis through effects on the intake, digestion, absorption and assimilation of nutrients (Dubé and Brubaker, 2004). GLP-1 was first identified as a potent incretin hormone that stimulates glucose-dependent insulin secretion. It inhibits gastric empting and glucagon secretion, while promoting pancreatic β-cell growth and peripheral insulin sensitivity. In contrast to GLP-1, GLP-2 appears to affect nutrient homeostasis indirectly, primarily by acting within the intestinal mucosa to increase growth, digestive and absorptive capacity and barrier function. It also acts on extraintestinal tissues (lymph nodes, spleen, liver etc) to reduce bone resorption and in CNS to induce neuronal survival. When taken together, these actions support the concept that GLP-1 and GLP-2 act through a variety of different mechanisms to regulate nutrient homeostasis (Drucker, 2002). These peptides are therefore not only interesting from a scientific perspective, but are also attractive for poultry production. The physiology of GLP-1 and GLp-2 is intimately tied, as both peptides are co-synthesized from a common proglucagon precursor and are co-secreted from the intestinal endocrine L-cells. Thus, GLP-1 and GLP-2 share identical regulatory pathways of expression, processing and secretion. However, the regulation of GLPs secretion is of utmost importance to the understanding of GLP physiology. Studies of the profile of GLP release and its regulation have revealed that GLP secretion is highly complex, involving interactions between ingested nutrients and both endocrine and neural inputs. GLP-1 and GLP-2 producing enteroendocrine L-cell is an open-type intestinal epithelial cell that is in contact with the intestinal lumen indicating that it may react to luminal signals. The L-cell displays an aboral localization profile in which the greatest density is found in the distal ileum. Enteral nutrient ingestion is the primary stimulus for GLP secretion. Following a mixed-meal, GLP secretion is induced. Interestingly, GLP secretion depends on the specific nutrient composition of the meal, and there are clear

differences in the response to proteins, carbohydrates and lipids. A minimum nutrient intake threshold must be reached to induce GLP secretion, and that the extent of enteral nutrition and rate of nutrient delivery to the intestine are critical factors in this response.

Oral administration of mixed liquid meal proved that the release of GLP-1 into the circulation occurs in a biphasic manner, consisting of a rapid (within 10-15 minutes) early phase followed by a more prolonged (30-60 minutes) second phase (Herrmann *et al*., 1995). The distal location of most L-cells that produce GLP-1 and GLP-2 makes it unlikely that the rapid nutrient-stimulated increase in plasma levels of these peptides is due to a direct effect of nutrients on the L-cells. Indeed, studies in rodents clearly indicate that the vagus nerve, the neurotransmitter gastrin-releasing peptide and the hormone glucose-dependent insulinotropic peptide all contribute to the rapid release of GLP-1 and GLP-2 from distal intestinal L-cells in response to nutritional stimuli (Brubaker and Anini, 2003). In contrast, the second phase of peptide secretion probably results from a direct stimulation of the L-cells by digested nutrients (Roberge and Brubaker, 1991). Thus, nutrient-induced stimulatory signals are transmitted to intestinal L-cells indirectly, via neural and endocrine effectors, and also by direct interaction with these cells, to mediate the first and second phase, respectively, of GLP-1 and GLP-2 secretion. A neural/endocrine feedback loop may also control GLP secretion. Somatostanin (SS) is an inhibitory peptide secreted from the intestine in two forms, the mainly neural SS-14 and the endocrine SS-28 (Brubaker *et al*., 1990). Both forms of SS are inhibitory to GLP secretion , as seen in several *in vivo* and *in vitro* models of the L-cell (Brubaker, 1991; Hansen *et al*., 2000; 2004). Furthermore, both GLP-1 and GLP-2 act to increase SS secretion in the gut (Jia *et al*., 1994; Brubaker *et al*., 1997b; Hansen *et al*., 2004), thereby suggesting that GLP secretion may be self-limiting via a SS-dependent inhibitory mechanism. Additionally, GLP-1 may act to limit its own secretion from L-cell, as GLP-1 inhibits GLP-2 secretion from the isolated ileum (Hansen *et al*., 2004). It is interesting to speculate that these potential feedback mechanisms may be

involved in development of pulsatile GLP secretion under both basal and meal-stimulated conditions (Balks *et al*., 1997).

The overall hypotheses of this dissertation are that GLPs exist in chicken respond to nutritional stimuli, and GLPs can induce gastrointestinal growth and intestinal proliferation. The aim of this research was to systematically characterize and histological evaluation of the mechanism for the regulation of GLPs secretion in chicken intestine. Prior to this, only indirect evidence was available to suggest the nutritional regulation of GLPs in chicken.

The first experiment established the colocalization of GLP-1 and GLP-2 in chicken intestinal tract. L-cells showing immunoreactivity for both GLP-1 and GLP-2 showed comma-like or flask-like shape and were located in epithelium of crypts and lower part of intestinal villi. L-cells showing GLP-1-immunoreactivity only were found in epithelium of lower and middle parts of intestinal villi. The distributional pattern of GLPs may be due to differences in their secretory phase as well as actions. Perhaps, both GLP-1 and -2 secrete simultaneously at first and then only GLP-1 to perform their respective activities. This study advances new morphological data about the endocrine system of the chicken small intestine. Experiment 2 demonstrated that the quantity of feed intake had an influence on the distributional pattern and activity of both GLP-1 and GLP-2-immunoreactive cells in the chicken small intestine. The number of GLP cells increased significantly with the advancement of restricting the amount of feed supply ($p<0.05$) showing location differences. These morphological changes indicate that the volume of feed intake is one of signals which have an influence on the secretory mechanism of GLPs from L-cells in the chicken small intestine. To assess the effects of quality of feed (dietary protein) on GLPs in the chicken the third experiment was performed. The study showed that the quality of ingested feed (protein) had an impact on the activities of both GLP-1 and -2-immunoreactive cells. Weight gains were declined correspondingly with diminishing of dietary protein level. This fact further strengthens our conclusion from the second experiment that amount of specific nutrient ingestion have effects in the intestinal chicken

GLPs. I suggest, based on these data, that the nutrients are highly involved in regulating the physiological activities of GLPs in the chicken small intestine. To my best of knowledge, this nutritional response to GLPs has never been reported in chicken prior to this dissertation. The data presented in this dissertation shows that chicken possess functional GLPs signaling system that is responsive to nutrient ingestion. This research sets the foundation for several directions of future research. This research demonstrating the impact of nutrients on chicken intestinal GLPs suggests that these hormones could substantially modify the capacity of the gastrointestinal tract for nutrient absorption by affecting the total surface area of the absorptive compartment. GLPs may give the industry a tool to modify the timing of gastrointestinal tract growth to better suit the upcoming needs of the chicken. Significant advances could also be achieved in basic biomedical research. It has long been known that GLP-1, co-secreted with GLP-2, stimulates insulin secretion, but it has recently been reported that GLP-2 induces glucagon secretion (de Heer *et al*., 2007). Therefore, these gastrointestinal hormones impact not only the profile of nutrients absorbed from the gastrointestinal tract but may also indirectly affect their utilization in peripheral tissues. This will be an important area of research in the future in that it may allow a more complete understanding of the interactions between diet and potential downstream metabolic effects via insulin and other metabolic hormones. Finally, this research discovered an important aspect of GLPs that has not previously been reported in chicken. This dissertation research systematically characterize and morphologically evaluates the mechanisms for the regulation of GLPs secretion in chicken intestine. While it extends the knowledge of hormonal control of the gastrointestinal tract in chicken, it also adds crucial information to the larger body of work investigating the actions of GLPs. This dissertation research has contributed to the groundwork necessary to enable the use of glucagon-like peptides in improving the health and productivity of a diverse group of avian species.

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