Amino Acid Supplementation to Diet Influences the Activity of the L Cells in Chicken Small Intestine

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Glucagon-like peptide-1 (GLP-1) is secreted by L cells in the small intestine in response to food ingestion. The influence of supplementation to diet with the amino acids, methionine (Met) and lysine (Lys), on the L cells in chicken small intestine was investigated using immunohistochemical and morphometrical techniques. Many endocrine cells showing immunoreactivity for GLP-1 antiserum were observed in the control, crude protein (CP) 0%, CP 0%+Met and CP 0%+Lys groups. The GLP-1-immunoreactive cells in all the groups were “open-typed” endocrine cells as viewed under light and electron microscopes. Differences in the shape and distributional pattern of GLP-1-immunoreactive cells were not observed between the control and experimental groups. Frequencies of the occurrence of GLP-1-immunoreactive cells in the CP 0%+Met and CP 0%+Lys groups were significantly lower than that of the CP 0% group, but significant differences were not recognized between the control group and the CP 0%+Met and CP 0%+Lys groups. Secretory granules in the control group were round to oval in shape. Elongated secretory granules were observed in the experimental groups, but not in the control group. Ratios of GLP-1-immunoreactive cells with elongated secretory granules in the CP 0%+Met and CP 0%+Lys groups were decreased compared with that in the CP 0% group. The size of round secretory granules in the control group was larger than that in all the groups. However, sizes of round secretory granules in the CP 0%+Met and CP 0%+Lys groups were larger than that in the CP 0% group. These morphological results indicate that amino acids may be a signal that influences on the secretion of GLP-1 in chicken small intestine.

Keywords: amino acid supplementation, chicken, glucagon-like peptide-1, immunohistochemistry, secretory granules, small intestine


Introduction

Glucagon-like peptide-1 (GLP-1) is a 30-amino acid peptide derived from the precursor, proglucagon, and it is secreted by L cells in the small intestinal epithelium in response to food ingestion (Balkan, 2000; Drucker, 2006). GLP-1 is identified as an “incretin hormone” with potent insulin-releasing activity, and it also decelerates gastric emptying (Nauck, 1998; Schirra et al., 2006). Immuno-histochemical and morphometrical studies in chicken intestine have shown that cells immunoreactive for GLP-1 anti-serum, L cells, are mainly distributed in the whole jejunum and ileum, and rarely found in the ascending duodenum (Hiramatsu et al., 2003, 2005). L cells in chicken small intestine are open-typed endocrine cells, which are apically covered with microvilli, and they store many secretory granules in their perikarya (Nishimura et al., 2013). These ultrastructural features indicate that the secretion of GLP-1 is induced by the presence of ingested feed in chicken small intestine. In fact, restricted feeding and dietary protein levels influence the frequency of the occurrence of GLP-1-immunoreactive cells in chicken small intestine (Monir et al., 2014a, b). It is expected that proteins are digested to the amino acid level in the lower small intestine of a chicken where many L cells are distributed. However, whether amino acids influence the secretion of GLP-1 from the L cells in chicken small intestine remains unclear.

In this study, we aimed to clarify the influence of essential
amino acids, methionine (Met) and lysine (Lys), on GLP-1-containing endocrine cells in chicken small intestine using immunohistochemical and morphometrical techniques.

Materials and Methods

Experimental Birds
Animal care was in compliance with applicable guidelines from the Iwate University Policy on Animal Care and Use. Male White Leghorn chicks \( (n = 20, \text{ seven days old}) \) that weighed 94.25 g on average were used in this study. They were evenly divided into four groups of five birds each; one control group and three experimental groups, including crude protein (CP) 0%, CP 0% + Met and CP 0% + Lys groups. For pre-experimental feeding, chicks were then forcedly fed a commercial chick mash diet three times a day for one day. After pre-experimental feeding, they were forcedly fed with the experimental diet three times a day for two days. The composition of each experimental diet is shown in Table 1.

Tissue Samples
After feeding the experimental diet, chicks were sacrificed by decapitation under anesthesia using diethylether. An approximately 3 cm long ileum was rapidly dissected from each bird, washed with 0.75% NaCl solution and immersed in 4% paraformaldehyde solution for 24 h at 4°C. Tissue samples were embedded in paraffin wax according to the standard procedures.

Immunohistochemistry
Paraffin sections were cut at 5 μm thickness and used for the immunohistochemical detection of GLP-1. The streptavidin-biotin method (Guesdon et al., 1979) was applied to detect GLP-1-immunoreactive cells according to the procedure previously described (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 1:2,000) was used as the primary antibody in this study. This antiserum did not cross-react with other proglucagon-derived peptides (Tachibana et al., 2005). After counterstaining with Mayer’s hematoxylin, sections were coverslipped and observed under a light microscope. To evaluate the villous height, the villous height of a total of 100 villi was measured in each group. The frequency of the occurrence of GLP-1-immunoreactive cells in the ileum was calculated using a computerized image analyzing system (KS400, ZEISS, Göttingen, Germany), according to the previously described method (Hiramatsu et al., 2005). Thirty areas were measured in each bird, and a total of 150 areas were measured from five chicks in each group. Statistical analyses were conducted to assess the difference in the frequency of the occurrence of GLP-1-containing cells among the four groups using Scheffe’s F test as a multiple comparison procedure.

Immunocytochemistry
Tissue samples embedded in paraffin wax were used for this approach. The pre-embedding technique for immunoelectron microscopy reprocessed from paraffin sections stained by the linked streptavidin-biotin method was applied (Watanabe et al., 2014). After detecting GLP-1-immunoreactive cells, sections were immersed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 15 min, post-fixed in 1% osmium tetroxide for 15 min, dehydrated through graded series of ethanol and embedded in epoxy resin (Quetol 812, Nissin EM, Tokyo, Japan). After polymerization of the resin, sections were removed from glass slides by warming them. Ultra-thin sections were cut using an ultramicrotome (Super Nova, Reichert-Jung, Vienna, Austria): they were then mounted on 200-mesh copper grids, stained with 10% (v/v) TI Blue (Nissin EM) and 2% (w/v) lead citrate and observed under a transmission electron microscope (JEM-1400, JEOL, Tokyo, Japan).

Morphometrical analysis was conducted to characterize the secretory granules in GLP-1-immunoreactive cells. The minimum and maximum diameters of all round secretory granules contained in a GLP-1-immunoreactive cell were measured using an image analyzer (KS400, ZEISS). Secretory granules were measured in 10 GLP-1-immunoreactive cells from each bird. Statistical analysis was con-

<table>
<thead>
<tr>
<th>Composition</th>
<th>Control</th>
<th>CP 0%</th>
<th>CP 0% + Met</th>
<th>CP 0% + Lys</th>
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<tr>
<td>ISP (CP 84%)</td>
<td>239.0</td>
<td>0.0</td>
<td>0.0</td>
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<tr>
<td>L-Methionine</td>
<td>2.9</td>
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<td>0.0</td>
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</tr>
<tr>
<td>Glycine</td>
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<td>0.0</td>
<td>0.0</td>
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<tr>
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<td>752.7</td>
<td>749.7</td>
<td>744.2</td>
</tr>
<tr>
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<td>154.3</td>
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<tr>
<td>Corn oil</td>
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<tr>
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<tr>
<td>Vitamin mixture</td>
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<tr>
<td>Choline chloride</td>
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<tr>
<td>Inositol</td>
<td>1.0</td>
<td>1.0</td>
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Diets are composed at the same energy density (metabolizable energy = 3,000 kcal/kg). CP: crude protein, ISP: isolated soybean protein, Met: methionine, Lys: lysine.
ducted using Scheffe’s F test for multiple comparisons.

Results

Table 2 summarizes the average body weight gain (g/2 days) and villous height (μm) of the four groups. Body weight gain and villous height in the control group were significantly higher than those in the three experimental groups.

Many endocrine cells showing immunoreactivity for GLP-1 antiserum were observed in all the groups. GLP-1-immunoreactive cells were mainly observed in the lower part of the intestinal villi and crypts in all the groups (Fig. 1a–d), with pyramidal or spindle-like shapes in the villous epithelium and comma-like shapes in the crypts. These cells had cytoplasmic processes reaching to the intestinal lumen (Fig. 1e–h). Obvious differences were not seen in the shapes or distributional patterns of GLP-1-immunoreactive cells among the four groups. The frequencies of the occurrence of GLP-1-immunoreactive cells in each group are summarized in Fig. 2. The frequencies of GLP-1-immunoreactive cells in the control, CP 0%, CP 0%+Met and CP 0%+Lys groups were 18.0±6.0, 23.5±7.8, 18.7±6.2 and 20.3±6.0, respectively (cell number per mucosal area: cells/mm², mean ±SD). The CP 0% group had a significantly higher value

Table 2. Average body weight gain and villous height of the four groups

<table>
<thead>
<tr>
<th></th>
<th>Body weight gain (g/2 days)</th>
<th>Villous height (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.0±4.7a</td>
<td>344±51a</td>
</tr>
<tr>
<td>CP 0%</td>
<td>5.3±3.1b</td>
<td>320±35b</td>
</tr>
<tr>
<td>CP 0%+Met</td>
<td>5.9±4.2b</td>
<td>312±38b</td>
</tr>
<tr>
<td>CP 0%+Lys</td>
<td>6.0±4.0b</td>
<td>321±28b</td>
</tr>
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Values are shown by mean±SD. a,b Values with different superscripts in the same column are significantly different (P<0.01).

Fig. 1. Glucagon-like peptide (GLP)-1-immunoreactive cells in chicken ileum from the control (a, e), CP 0% (b, f), CP 0%+Met (c, g) and CP 0%+Lys (d, h) groups. a–d. Obvious differences are not observed in the distributional patterns of GLP-1-immunoreactive cells among the four groups. GLP-1-immunoreactive cells are mainly distributed in the lower part of the intestinal villi and crypts. Bars: 100μm. e–h. GLP-1-immunoreactive cells at a higher magnification. They get a contact with intestinal lumen by the cytoplasmic process. Obvious differences are not seen in the shapes of GLP-1-immunoreactive cells among the four groups. Bar: 10μm.
than that of the other three groups.

At a lower magnification of the electron microscope, GLP-1-immunoreactive cells were easily identified by the dark reaction products on secretory granules (Fig. 3a, b). GLP-1-immunoreactive cells were flask-like or comma-like in shape with an oval nucleus. They had cytoplasmic processes apically covered with microvilli (Fig. 3a, b). Differences in cell shape were not seen among the four groups.

The secretory granules accumulated mainly in the basal cytoplasm or in the perinuclear region of GLP-1-immunoreactive cells. The secretory granules were observed in all the groups; and many of them were round to oval in shape (Fig. 3c). A few elongated granules were also observed in the CP 0%, CP 0%+Met and CP 0%+Lys groups (Fig. 3d), but not in the control group. The major axis of these elongated granules was 1–2 μm. The ratio of GLP-1-immunoreactive cells that contained elongated granules (elongated granules-containing cells/GLP-1-immunoreactive cells) in the control, CP 0%, CP 0%+Met and CP 0%+Lys groups were calculated and they were found to be 0% (0/50), 17% (9/52), 4% (3/68) and 11% (10/92), respectively. The ratio of GLP-1-immunoreactive cells that contained elongated granules was highest in the CP 0% group, but it decreased with amino acid supplementations. The results of the morphometry of round secretory granules are shown in Table 3. The minimum and maximum diameters of round secretory granules were largest in the control group, and they became smaller in the following order: CP 0%+Lys>CP 0%+Met>CP 0%. The size of round secretory granules in the CP 0% group was the smallest among all groups, and showed a significant difference from the other three groups. No significant difference was seen between the CP 0%+Lys and CP 0%+Met groups.

**Discussion**

The present study demonstrated that the amino acids Met and Lys influenced the activity of GLP-1-immunoreactive cells, namely L cells, in chicken small intestine. Our previous studies in chicken small intestine showed that the L cells were open-typed endocrine cells that were covered apically with microvilli (Nishimura et al., 2013) and they were influenced by protein ingestion (Monir et al., 2014a). Protein is digested to amino acids and absorbed by enterocytes of the small intestine where the L cells are distributed (Hiramatsu et al., 2003, 2005). These findings led to the hypothesis that amino acids were one of the triggers that stimulate GLP-1 secretion from the L cells in chicken small intestine. In this study, frequencies of the occurrence of GLP-1-immunoreactive cells were decreased in the Met and Lys-added groups compared with those in the CP 0% group. This data indicated that Met and Lys stimulated the secretion of GLP-1 from the ileal L cells resulting in a decrease in cells showing immunoreactivity for GLP-1 antiserum.

In the mammalian intestine, it has been shown that protein and amino acids ingestion could stimulate GLP-1 release from the intestinal L cells. The intraduodenal infusion of protein increased the plasma concentration of GLP-1 in lean, healthy men (Ryan et al., 2012). Glutamine is a potent stimulus of GLP-1 secretion both in vivo and in vitro (Reimann et al., 2004; Greenfield et al., 2009). Oral intake of L-arginine also stimulates GLP-1 secretion in male mice (Clemmensen et al., 2013). These reports support the concept that amino acid may be a signal that influences the secretion of GLP-1 in chicken small intestine. Several studies have revealed that cholecystokinin is secreted from enteroendocrine cells in response to protein hydrolysates.
peptides and amino acids (Foltz et al., 2008; Nakajima et al., 2010; Daly et al., 2013). Thus, amino acids are one of the important stimuli for intestinal hormone secretion in both mammals and chickens.

In this study, we observed elongated secretory granules in GLP-1-immunoreactive cells in the CP 0% group. Such secretory granules were stored at relatively higher ratios in the CP 0% group (about 17%), but not in the control group. This ratio decreased with Met and Lys supplementation. Moreover, the size of round secretory granules in GLP-1-immunoreactive cells was smallest in the CP 0% group followed by that in the CP 0%+Met, CP 0%+Lys and control groups (in ascending order). These findings indicate amino acid involvement in the secretory process of GLP-1 in the L cells of chicken ileum.

A decrease in hormone secretion stimuli induces various ultrastructural changes, such as the appearance of vacuoles in endocrine cells of the pituitary gland and pancreatic islets (Smith and Farquhar, 1966; Borg and Schnell, 1986; Skoglund et al., 1987). These changes are regarded as physiological phenomena to protect against decrease in stimuli to hormone secretion; this is called crinophagy. Crinophagy is the intracellular digestion by lysosomes to clean up redundant secretory granules that are stored in endocrine cells because of no secretory stimuli. Crinophagy is known to be the major mechanism by which the peptide-secreting endocrine cells degrade excess secretory material (Marzella et al., 1981). The elongated secretory granules in the CP 0% group may be granules that have been digested by lysosomes. It is possible that crinophagy is induced in the L cells under protein-free conditions; however, systematic studies are necessary to clarify this phenomenon.

In conclusion, amino acids are a signal that influences GLP-1 secretion of the L cells in chicken small intestine.

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References

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