Effects of Dietary Crude Protein Level on Calcium-Sensing Receptor Gene Expression, Gastrointestinal Hormone Secretion, and Gastric Functional Enzyme Activity in Pigs: Postprint

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Abstract

This experiment aimed to investigate the effects of different dietary crude protein levels on calcium-sensing receptor (CaSR) gene expression in the gastrointestinal tract, gastrointestinal hormone secretion, and gastric functional enzyme (H+-K+-ATPase, pepsin) activities in pigs, and to explore the relationships between small intestinal CaSR gene expression levels and serum gastrointestinal hormone concentrations, as well as between gastric CaSR gene expression levels and H+-K+-ATPase and pepsin activities. Eighteen crossbred weaned piglets (Duroc × Landrace × Large White) at 35 days of age with an initial body weight of (9.57±0.64) kg were selected and randomly divided into 3 groups: NP group (NRC standard crude protein level group), MP group (3% lower crude protein level than the standard protein level group), and LP group (6% lower crude protein level than the standard crude protein level group), with 6 replicates per group and 1 piglet per replicate. According to the nutritional requirements of pigs at different growth stages, diets with crude protein levels of 20% (NP group), 17% (MP group), and 14% (LP group) were fed during the piglet stage (35–80 days of age), diets with crude protein levels of 18% (NP group), 15% (MP group), and 12% (LP group) were fed during the growing pig stage (81–110 days of age), and diets with crude protein levels of 16% (NP group), 13% (MP group), and 10% (LP group) were fed during the finishing pig stage (111–160 days of age). The dietary levels of lysine (Lys), methionine (Met), threonine (Thr), and tryptophan (Trp) were balanced across treatments. The experimental period lasted 125 days. At the end of the experiment, blood was collected from the anterior vena cava, and after slaughtering all experimental pigs, gastric chyme, stomach, duodenum, jejunum, and ileum tissues and mucosa were collected to determine serum gastrointestinal hormone concentrations, pepsin

activity in gastric chyme and H+-K+-ATPase activity in gastric mucosa, as well as CaSR gene expression levels in various segments of the gastrointestinal tract. The results showed that: 1) Gastric CaSR gene expression level in the LP group was significantly higher than that in the NP and MP groups ($P<0.05$), while duodenal and jejunal CaSR gene expression levels in both MP and LP groups were significantly lower than those in the NP group ($P<0.05$). However, there was no significant difference in ileal CaSR gene expression levels among all groups ($P>0.05$). 2) Compared with the NP group, serum peptide YY (PYY) and glucose-dependent insulinotropic peptide (GIP) concentrations in pigs from MP and LP groups were significantly decreased ($P<0.05$), serum cholecystokinin (CCK) concentration in the MP group was significantly increased ($P<0.05$), and H+-K+-ATPase activity in gastric mucosa and pepsin activity in gastric chyme in the LP group were significantly increased ($P<0.05$). 3) Gastric CaSR gene expression level was significantly positively correlated with H+-K+-ATPase activity in gastric mucosa and pepsin activity in gastric chyme ($P<0.05$). Duodenal CaSR gene expression level was significantly positively correlated with serum GIP concentration ($P<0.05$) and showed a trend toward significant positive correlation with serum PYY concentration ($0.05 P<0.10$). Jejunal CaSR gene expression level was significantly positively correlated with both serum GIP and PYY concentrations ($P<0.05$). Ileal CaSR gene expression level showed a trend toward significant positive correlation with serum PYY concentration ($0.05 P<0.10$). In conclusion, reduced dietary crude protein levels affected CaSR gene expression in the gastrointestinal tract of pigs, thereby influencing gastric functional enzyme activities and gastrointestinal hormone secretion.

Full Text

Effects of Dietary Crude Protein Level on Calcium Sensing Receptor Gene Expression in the Gastrointestinal Tract, Gastrointestinal Hormone Secretion, and Gastric Functional Enzyme Activities in Pigs

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Abstract: This experiment was conducted to investigate the effects of different dietary crude protein (CP) levels on calcium sensing receptor (CaSR) gene expression in the gastrointestinal tract, gastrointestinal hormone secretion, and the activities of gastric functional enzymes (H+-K+-ATPase and pepsin) in pigs, and to explore the relationships between CaSR gene expression in the small intestine and serum gastrointestinal hormone concentrations, as well as between gastric CaSR gene expression and the activities of H+-K+-ATPase and pepsin. Eighteen 35-day-old "Duroc × Landrace × Large White" crossbred weaned

piglets with an initial body weight of (9.57 ± 0.64) kg were randomly divided into three groups: NP group (NRC standard CP level), MP group (3% lower CP than the standard), and LP group (6% lower CP than the standard), with six replicates per group and one piglet per replicate. According to the nutritional requirements of pigs at different growth stages, diets containing 20% (NP group), 17% (MP group), and 14% (LP group) CP were fed during the weanling period (35–80 days of age); diets containing 18% (NP group), 15% (MP group), and 12% (LP group) CP were fed during the growing period (81–110 days of age); and diets containing 16% (NP group), 13% (MP group), and 10% (LP group) CP were fed during the finishing period (111–160 days of age). The dietary levels of lysine (Lys), methionine (Met), threonine (Thr), and tryptophan (Trp) were balanced across treatments. The experimental period lasted for 125 days. At the end of the experiment, precaval vein blood was collected, and after slaughter, gastric chyme, stomach, duodenum, jejunum, and ileum tissues and mucosa were sampled to determine serum gastrointestinal hormone concentrations, gastric pepsin and mucosal H+-K+-ATPase activities, and CaSR gene expression levels in various gastrointestinal segments.

The results showed: 1) Gastric CaSR gene expression in the LP group was significantly higher than in the NP and MP groups ($P < 0.05$), while duodenal and jejunal CaSR gene expression in the MP and LP groups were significantly lower than in the NP group ($P < 0.05$). No significant differences were observed in ileal CaSR gene expression among groups ($P > 0.05$). 2) Compared with the NP group, serum peptide tyrosine tyrosine (PYY) and glucose-dependent insulinotropic peptide (GIP) concentrations were significantly reduced in the MP and LP groups ($P < 0.05$), serum cholecystokinin (CCK) concentration was significantly elevated in the MP group ($P < 0.05$), and gastric mucosal H+-K+-ATPase and gastric pepsin activities were significantly increased in the LP group ($P < 0.05$). 3) Gastric CaSR gene expression was significantly positively correlated with both gastric mucosal H+-K+-ATPase activity and gastric pepsin activity ($P < 0.05$). Duodenal CaSR gene expression was significantly positively correlated with serum GIP concentration ($P < 0.05$) and showed a positive correlation trend with serum PYY concentration ($0.05 \quad P < 0.10$). Jejunal CaSR gene expression was significantly positively correlated with both serum GIP and PYY concentrations ($P < 0.05$). Ileal CaSR gene expression showed a positive correlation trend with serum PYY concentration ($0.05 \quad P < 0.10$).

In conclusion, reducing dietary crude protein level affects CaSR gene expression in the porcine gastrointestinal tract, thereby influencing gastric functional enzyme activities and gastrointestinal hormone secretion.

Keywords: pigs; crude protein level; calcium sensing receptor; gastrointestinal hormone; H+-K+-ATPase; pepsin

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The gastrointestinal tract not only digests and absorbs nutrients from feed but also secretes gastrointestinal hormones, playing a crucial role in maintaining di-

gestive function and homeostasis. Various endocrine cells are scattered throughout the gastrointestinal tract, including gastric parietal cells, D cells, and antral G cells [1], which express multiple nutrient-sensing receptors [2] that detect carbohydrates, fatty acids, amino acids, and peptides, thereby regulating gastrointestinal hormone secretion and influencing digestive and absorptive functions [3]. The calcium sensing receptor (CaSR) gene can sense amino acids and peptides, particularly aromatic L-amino acids such as L-phenylalanine (L-Phe) and L-tryptophan (L-Try) [4–8], and is widely expressed in gastrointestinal endocrine cells [3–4].

Both CaSR and H+-K+-ATPase are expressed in gastric parietal cells, and H+-K+-ATPase activity is regulated by CaSR. Busque et al. [9] found that CaSR in rat gastric parietal cells can mediate amino acids to enhance H+-K+-ATPase activity related to gastric acid secretion. Mace et al. [10] demonstrated that L-amino acids can activate CaSR, promoting the secretion of glucose-dependent insulinotropic peptide (GIP), peptide tyrosine tyrosine (PYY), and glucagon-like peptide-1 (GLP-1) in cultured rat small intestine. Liou et al. [11] in studies of mouse I cells and Hira et al. [12] in studies of mouse STC-1 cells both found that L-Phe can activate CaSR to promote cholecystokinin (CCK) secretion. To date, research on the effects of protein and amino acids on CaSR gene expression and function in the gastrointestinal tract has primarily focused on humans, mice, and rats, with most studies being in vitro experiments. In vivo studies in pigs have not been reported. Therefore, this experiment was designed to investigate the effects of long-term feeding of diets with different crude protein levels on CaSR gene expression in the porcine gastrointestinal tract, gastrointestinal hormone secretion, and gastric functional enzyme activities.

1.1 Experimental Animals and Design

Eighteen 35-day-old "Duroc × Landrace × Large White" crossbred weaned piglets with an initial body weight of (9.57 ± 0.64) kg were randomly divided into three groups based on dietary crude protein level: NP group (NRC standard crude protein level), MP group (3% lower crude protein than the standard), and LP group (6% lower crude protein than the standard). Each group had six replicates with one piglet per replicate, housed individually with ad libitum access to water and feed. Corn-soybean meal-based basal diets were formulated according to NRC standards (2012). After a 3-day pre-feeding period, pigs were fed diets containing 20% (NP group), 17% (MP group), and 14% (LP group) crude protein during the weanling period (35–80 days of age); 18% (NP group), 15% (MP group), and 12% (LP group) crude protein during the growing period (81–110 days of age); and 16% (NP group), 13% (MP group), and 10% (LP group) crude protein during the finishing period (111–160 days of age). Dietary lysine (Lys), methionine (Met), threonine (Thr), and tryptophan (Trp) levels were balanced across treatments. The experimental period lasted for 125 days. Dietary composition and nutrient levels are shown in Table 1 . The experiment was conducted at the Pig Metabolism Laboratory of the Institute of Subtropical Agriculture,

Chinese Academy of Agricultural Sciences. Before the experiment, pig house environments and equipment were cleaned, fumigated, and disinfected. During the trial, regular disinfection, deworming, and immunization were performed.

1.2 Sample Collection

1.2.1 Serum Samples All experimental pigs were fasted for 24 hours before the end of the experiment with free access to water. On day 125, 100 mL of precaval vein blood was collected from each pig. After coagulation, serum was obtained by centrifugation and stored at -20 °C for determination of gastrointestinal hormone CCK, GIP, and PYY concentrations.

1.2.2 Gastrointestinal Samples After blood collection, pigs were slaughtered and the abdominal cavity was opened to immediately remove the entire digestive tract and separate and ligate each segment. Gastric chyme was thoroughly mixed and quickly collected, then stored at -20 °C for pepsin activity analysis. Stomach, duodenum, jejunum, and ileum tissues and mucosa were excised on ice, rinsed in phosphate-buffered saline (PBS) to remove contents, and stored in liquid nitrogen for H+-K+-ATPase activity analysis and CaSR gene expression detection.

1.3 Laboratory Analyses

1.3.1 Serum Gastrointestinal Hormone Concentrations Enzyme-linked immunosorbent assay (ELISA) was used to detect gastrointestinal hormone CCK, PYY, and GIP concentrations in precaval vein serum. Porcine CCK (FU-Z044; CCK8 antibody, orb10260, biorbyt), PYY (FU-Z240, PYY antibody, LS-C191185-400, LifeSpan BioSciences), and GIP (FU-A192; GIP antibody GTX37687 GeneTex) assay kits were purchased from Beijing Fangcheng Biological Technology Co., Ltd.

1.3.2 Gastric Functional Enzyme Activities Gastric pepsin activity in chyme and H+-K+-ATPase activity in gastric mucosa were strictly measured according to kit instructions. Pepsin (A080-1) and H+-K+-ATPase (A069) assay kits were purchased from Nanjing Jiancheng Yuehao Technology Co., Ltd.

1.3.3 CaSR Gene Expression Levels Total RNA was extracted from each gastrointestinal segment according to the method of Chomczynski et al. [13] and reverse-transcribed following the kit protocol. Primers were designed based on porcine CaSR sequence (NM_001278748) and porcine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) sequence (NM_001206359.1) from NCBI. CaSR primer sequences: forward 5' -CGGGGGACTCTTTCCTATTC-3' , reverse 5' -GCTGGGCTGCTGTTTATTTC-3' , annealing temperature 60 °C; GAPDH primer sequences: forward 5' -ATGGTGAAGGTCGGAGTGAAC-3' , reverse 5' -GCTGGGCTGCTGTTTATTTC-3' , annealing temperature 60 °C. Fluorescence quantitative PCR analysis of total RNA extracted from porcine

gastrointestinal segments was performed according to reference [14]. The 2^(-ΔΔCt) method was used to calculate relative CaSR gene expression levels. Total RNA extraction kit (RNAiso Plus, 9108), reverse transcription kit (RT reagent Kit with gDNA Eraser, RR047A), and fluorescence quantitative PCR kit (SYBR® Premix Ex TaqTM, RR420A) were purchased from Takara Bio (Dalian) Co., Ltd.

1.4 Statistical Analysis

Experimental data were initially organized using Excel 2010 and analyzed using one-way ANOVA in SPSS 20.0 software. Significant differences were tested using S-N-K test, with $P < 0.05$ considered significant and $P < 0.01$ considered highly significant. Correlations between CaSR gene expression levels in gastrointestinal segments and H+-K+-ATPase activity, pepsin activity, and serum gastrointestinal hormone concentrations were analyzed using GraphPad Prism 5. $P < 0.05$ was considered significantly correlated, and 0.05 $P < 0.10$ indicated a correlation trend.

2 Results

2.1 Effects of Dietary Crude Protein Level on CaSR Gene Expression in Porcine Gastrointestinal Tract

As shown in Figure 1 [Figure 1: see original paper], gastric CaSR gene expression in the LP group was significantly higher than in the NP and MP groups ($P < 0.05$), with no significant difference between NP and MP groups ($P > 0.05$). In the duodenum and jejunum, CaSR gene expression in MP and LP groups was significantly lower than in the NP group ($P < 0.05$), with no significant difference between MP and LP groups ($P > 0.05$). However, no significant differences were observed in ileal CaSR gene expression among all groups ($P > 0.05$).

2.2 Effects of Dietary Crude Protein Level on Serum Gastrointestinal Hormone Concentrations and Gastric Functional Enzyme Activities

As shown in Table 2 , compared with the NP group, serum PYY and GIP concentrations were significantly reduced in MP and LP groups ($P < 0.05$), with no significant difference between MP and LP groups ($P > 0.05$). Serum CCK concentration was significantly elevated in the MP group ($P < 0.05$), while no significant change was observed in the LP group ($P > 0.05$). Gastric mucosal H+-K+-ATPase activity and gastric pepsin activity were significantly increased in the LP group ($P < 0.05$), with no significant changes in the MP group ($P > 0.05$).

2.3 Correlation Analysis Between CaSR Gene Expression and Serum Gastrointestinal Hormone Concentrations and Gastric Functional Enzyme Activities

As shown in Table 3 , gastric CaSR gene expression was significantly positively correlated with both gastric mucosal H+-K+-ATPase activity and gastric pepsin activity ($P < 0.05$). Duodenal CaSR gene expression was significantly positively correlated with serum GIP concentration ($P < 0.05$), showed no significant correlation with serum CCK concentration ($P > 0.05$), and exhibited a positive correlation trend with serum PYY concentration ($0.05 \quad P < 0.10$). Jejunal CaSR gene expression was significantly positively correlated with both serum GIP and PYY concentrations ($P < 0.05$) but not with serum CCK concentration ($P > 0.05$). Ileal CaSR gene expression showed no significant correlation with serum CCK and GIP concentrations ($P > 0.05$) but exhibited a positive correlation trend with serum PYY concentration ($0.05 \quad P < 0.10$).

3 Discussion

CaSR is widely expressed in various parts of the digestive tract in rodents, including the esophagus, stomach, and small intestine [15]. This study found that CaSR is also expressed in the stomach and all segments of the small intestine in pigs. The degradation products of dietary crude protein are mainly small peptides and amino acids [2], which can activate CaSR [10,16]. Conigrave et al. [17-18] found that aromatic amino acids can modulate CaSR activity when extracellular calcium is at a certain concentration, and CaSR gene expression increases with rising amino acid concentrations. This study revealed that when dietary crude protein level decreased, gastric CaSR gene expression increased, possibly because the balanced four amino acids (Lys, Met, Thr, and Trp) in the diet included Trp, which can activate CaSR. As dietary crude protein level decreased, Trp supplementation increased. Since dietary crude protein had not yet been degraded into amino acids in the stomach, higher levels of supplemental Trp exerted stronger activation of CaSR, leading to increased CaSR gene expression. Conversely, duodenal and jejunal CaSR gene expression decreased with reduced dietary crude protein levels, likely because peptides and amino acids produced from protein digestion in the stomach activated CaSR in the duodenum and jejunum, increasing its expression. Although ileal CaSR gene expression slightly decreased with reduced crude protein levels, the change was not significant, possibly because most amino acids had already been digested and absorbed in the duodenum and jejunum, resulting in low amino acid concentrations reaching the ileum that were insufficient to regulate CaSR gene expression.

Studies have shown that CaSR is highly expressed in gastric parietal cells [19-20], and L-aromatic amino acids can activate CaSR to enhance H+-K+-ATPase and pepsin activities [9,21]. In this study, as dietary crude protein level decreased and Trp supplementation increased, pepsin and H+-K+-ATPase activities increased. Correlation analysis revealed that increased gastric CaSR gene expression was accompanied by elevated H+-K+-ATPase and pepsin activities, with a

significant positive correlation, consistent with previous findings. Research also indicates that increased crude protein intake enhances secretion of hormones such as GIP and PYY in humans [22]. This study showed that reduced dietary crude protein level decreased secretion of gastrointestinal hormones PYY and GIP. Studies have demonstrated that amino acids promote secretion of GIP, CCK, and PYY in rat small intestine, with the CaSR-specific agonist NPS R568 significantly enhancing this effect, while the CaSR-specific inhibitor Calhex 231 significantly suppressing it [11]. Accordingly, this experiment analyzed correlations between porcine small intestinal CaSR gene expression and gastrointestinal hormones. The results showed that, except for CCK, other gastrointestinal hormones were significantly or tendentiously positively correlated with CaSR gene expression in the duodenum and ileum. When dietary crude protein level decreased, reduced amino acid levels from protein hydrolysis in the small intestine decreased CaSR sensitivity to amino acids, leading to reduced gastrointestinal hormone secretion. These results preliminarily indicate that amino acids can regulate gastrointestinal hormone secretion and gastric functional enzyme activities by activating CaSR. Shi et al. [23] and Leray et al. [24] reported that reduced dietary crude protein level promotes CCK release in the gastrointestinal tract. In this study, a 3% reduction in protein level decreased small intestinal CaSR gene expression but increased CCK secretion, possibly representing a compensatory response to insufficient nutrient intake to maintain homeostasis under low protein conditions. Currently, due to the complex in vivo environment and numerous influencing factors, few studies have examined the effects of protein and its hydrolysis products on gastrointestinal hormone secretion via CaSR in vivo. Therefore, our research group plans to use in vitro perfusion techniques in subsequent studies to investigate the relationship between porcine gastrointestinal CaSR gene expression and gastrointestinal hormone secretion, and to employ immunohistochemistry and immunofluorescence techniques to explore the signaling pathways through which amino acids activate CaSR to regulate gastrointestinal hormone secretion and gastric functional enzyme activities, thereby revealing the relationship and mechanisms linking porcine gastrointestinal CaSR gene expression with gastrointestinal hormone secretion and gastric functional enzyme activities.

In summary, reducing dietary crude protein level affects CaSR gene expression in the porcine gastrointestinal tract, thereby influencing the secretion of certain gastrointestinal hormones (GIP, PYY) and the activities of gastric functional enzymes (pepsin, H+-K+-ATPase).

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Note: Figure translations are in progress. See original paper for figures.