Effects of Dietary Crude Protein Level on Hepatic Amino Acid Metabolic Enzyme Activity and Transporter mRNA Expression in Weaned Piglets (Postprint)

Authors: Zhang Xiangxin, Chen Cheng, Tang Zhiru, Zhen Jifu, Xu Qingqing, Sun Zhihong

Date: 2018-12-24T00:00:00+00:00

Abstract

This experiment aimed to investigate the effects of dietary crude protein (CP) level on hepatic amino acid metabolic enzyme activities and transporter mRNA expression in weaned piglets. A total of 54 healthy Duroc × Landrace × Yorkshire crossbred weaned piglets at 28 days of age with similar body weight [(7.0±0.5) kg] (half male and half female) were randomly assigned to 3 groups [20% CP group (control group), 17% CP group, and 14% CP group], with 18 replicates per group and 1 pig per replicate. The experiment consisted of a 7-day preliminary period and a 45-day formal experimental period. On days 10, 25, and 45 of the formal experimental period, 6 piglets were selected from each group for slaughter. The results showed: 1) On day 10 of the experiment, hepatic glutamic-oxaloacetic transaminase (GOT) activity in weaned piglets from the 14% and 17% CP groups was significantly lower than that in the control group ($P<0.05$); hepatic glutamine synthetase (GS) activity in the 14% CP group was significantly lower than that in the other two groups ($P<0.05$). On day 25, hepatic GOT and GS activities in the 14% CP group were significantly lower than those in the control group ($P<0.05$); hepatic glutamic-pyruvic transaminase (GPT) and glutamate dehydrogenase (GDH) activities in the 14% and 17% CP groups were significantly lower than those in the control group ($P<0.05$). On day 45, hepatic GPT and GS activities in the 14% CP group were significantly lower than those in the other two groups ($P<0.05$). 2) On day 25 of the experiment, the relative mRNA expression levels of hepatic solute carrier family 6 member 15 (SLC6A15) and solute carrier family 38 member 2 (SLC38A2) in weaned piglets from the 14% CP group were significantly lower than those in the control group ($P<0.05$); the relative mRNA expression level of hepatic solute carrier family 36 member 1 (SLC36A1) in the 14% and 17% CP groups

was significantly lower than that in the control group (P<0.05). On day 45, the relative mRNA expression levels of hepatic solute carrier family 6 member 20 (SLC6A20) and SLC38A2 in the 14% and 17% CP groups were significantly lower than those in the control group (P<0.05); the relative mRNA expression level of hepatic SLC6A15 in the 14% CP group was significantly lower than that in the other two groups (P<0.05). These results indicate that reducing dietary CP level by 3% and 6% can decrease hepatic amino acid metabolic enzyme activities and transporter mRNA expression levels in weaned piglets.

Full Text

Effects of Dietary Crude Protein Level on Activities of Amino Acid Metabolic Enzymes and mRNA Expression of Amino Acid Transporters in Liver of Weaned Piglets

ZHANG Xiangxin[1,2], CHEN Cheng[1,2], TANG Zhiru[1,2], ZHEN Jifu[1,2], XU Qingqing[1,2], SUN Zhihong[1,2]*

[1]Laboratory of Bio-Feed and Molecular Nutrition, Southwest University, Chongqing 400715, China
[2]College of Animal Science and Technology, Southwest University, Chongqing 400715, China

Abstract

This study investigated the effects of dietary crude protein (CP) level on activities of amino acid metabolic enzymes and mRNA expression of amino acid transporters in the liver of weaned piglets. Fifty-four healthy 28-day-old "Duroc × Landrace × Yorkshire"hybrid weaned piglets with similar body weight [(7.0±0.5) kg] were randomly allocated to three groups: 20% CP (control), 17% CP, and 14% CP, with 18 replicates per group and one pig per replicate. The experiment consisted of a 7-day pre-trial period followed by a 45-day formal trial period. On days 10, 25, and 45 of the formal trial, six piglets were selected from each group for slaughter.

The results showed: (1) On day 10, hepatic glutamic-oxaloacetic transaminase (GOT) activity in the 14% and 17% CP groups was significantly lower than in the control group (P<0.05), while hepatic glutamine synthetase (GS) activity in the 14% CP group was significantly lower than in the other two groups (P<0.05). On day 25, hepatic GOT and GS activities in the 14% CP group were significantly lower than in the control group (P<0.05), and hepatic glutamic-pyruvic transaminase (GPT) and glutamic acid dehydrogenase (GDH) activities in both the 14% and 17% CP groups were significantly lower than in the control group (P<0.05). On day 45, hepatic GPT and GS activities in the 14% CP group were significantly lower than in the other two groups (P<0.05). (2) On day 25, the mRNA relative expression levels of solute carrier family 6 member 15 (SLC6A15) and solute carrier family 38 member 2 (SLC38A2) in the 14%

CP group were significantly lower than in the control group (P<0.05), while the mRNA relative expression of solute carrier family 36 member 1 (SLC36A1) in both the 14% and 17% CP groups was significantly lower than in the control group (P<0.05). On day 45, the mRNA relative expression levels of solute carrier family 6 member 20 (SLC6A20) and SLC38A2 in the 14% and 17% CP groups were significantly lower than in the control group (P<0.05), and the mRNA relative expression of SLC6A15 in the 14% CP group was significantly lower than in the other two groups (P<0.05). In conclusion, reducing dietary CP level by 3% and 6% decreased the activities of amino acid metabolic enzymes and the mRNA relative expression levels of amino acid transporters in the liver of weaned piglets.

Keywords: low-protein diets; piglets; liver; amino acid metabolic enzyme; amino acid transporter

Introduction

Intensification of livestock production has led to increasingly severe nitrogen pollution. In China, annual nitrogen emissions from pig production reach approximately 1,800 tonnes. Therefore, improving protein utilization efficiency and reducing nitrogen emissions in pigs is of significant scientific and social importance. Low-protein diets represent a common technology for reducing nitrogen emissions in swine. Studies have shown that reducing dietary CP level by 1% can decrease nitrogen emissions by approximately 8% [1]. With supplementation of essential amino acids, dietary CP level can be reduced by 2-4% without affecting pig growth and development [2]. Our previous research found that reducing dietary CP level while balancing only key essential amino acids (EAAs) [lysine (Lys), methionine (Met), tryptophan (Trp), and threonine (Thr)] significantly increased EAA consumption in liver tissue [3]. This experiment was designed to investigate the effects of dietary CP level on activities of amino acid metabolic enzymes and mRNA expression of amino acid transporters in the liver of weaned piglets, providing a scientific basis for elucidating the mechanism by which low-protein diets increase EAA consumption and reduce nitrogen emissions, and for improving amino acid metabolic conversion efficiency in weaned piglet liver.

1.1 Experimental Design and Diets

Fifty-four healthy 28-day-old "Duroc × Landrace × Yorkshire" hybrid weaned piglets with similar body weight [(7.0±0.5) kg] were randomly divided into three groups: 20% CP (control), 17% CP, and 14% CP. Each group comprised 18 replicates with one pig per replicate. The 17% and 14% CP groups were supplemented with Lys, Met, Thr, and Trp to maintain levels equivalent to the

control group. The basal diets were formulated according to NRC (2012) standards. The composition and nutrient levels of experimental diets are presented in Table 1 .

1.2 Animal Management

The trial was conducted at the Southwest University Animal Research Facility. The experiment included a 7-day pre-trial period and a 45-day formal trial period. Piglets were housed individually in stainless steel cages (1.50 m × 0.68 m × 0.75 m). The room temperature was maintained at (25±1) °C. All piglets had ad libitum access to feed and water, with feeding times at 08:00 and 18:00 daily. Pens were kept clean and dry throughout the experimental period.

1.3 Sample Collection

During the trial, feed samples from each group were collected three times using the quartering method, mixed, ground to pass through a 40-mesh sieve, and stored at room temperature. Dietary dry matter, CP, calcium, phosphorus, crude fiber, and amino acid contents were analyzed according to *Feed Analysis and Feed Quality Detection Technology* [4]. On days 10, 25, and 45 of the formal trial, six piglets closest to the average body weight were selected from each group for slaughter. Liver samples were collected, snap-frozen in liquid nitrogen, and stored at -80 °C.

1.4.1 Hepatic Amino Acid Metabolic Enzyme Activities

Liver tissue (0.6-0.9 g) was placed in a 10 mL centrifuge tube containing ice-cold 0.9% saline solution at a tissue-to-solution ratio of 1:9 (w/v), then homogenized on ice. The homogenate was centrifuged at 3,000 r/min for 10 min at 4 °C, and the supernatant was collected and stored at -20 °C for subsequent enzyme activity assays. Hepatic activities of glutamic-pyruvic transaminase (GPT) (C009-2), glutamic-oxaloacetic transaminase (GOT) (C010-2), glutamine synthetase (GS) (A047), and glutamic acid dehydrogenase (GDH) (A125) were measured using colorimetric assay kits purchased from Nanjing Jiancheng Bioengineering Institute, following the manufacturer' s instructions strictly.

1.4.2 Hepatic Amino Acid Transporter mRNA Expression

1.4.2.1 RNA Extraction and cDNA Synthesis Frozen liver tissue stored at -80 °C was placed on ice and minced with sterile scissors. The tissue was ground thoroughly in liquid nitrogen to prevent degradation from temperature

increase. The powdered tissue was transferred to a centrifuge tube. Total RNA was extracted using Total RNA Extractor (Shanghai Bioengineering Co., Ltd.), and reverse transcription was performed using MMLV First Strand cDNA Synthesis Kit (Shanghai Bioengineering Co., Ltd.) to obtain cDNA.

1.4.2.2 Primer Design Primers were designed using Primer Premier 5.0 software and synthesized by Shanghai Bioengineering Co., Ltd. Primer sequences for amino acid transporters solute carrier family 6 member 15 (SLC6A15), solute carrier family 6 member 20 (SLC6A20), solute carrier family 36 member 1 (SLC36A1), solute carrier family 38 member 2 (SLC38A2), and the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are listed in Table 2 .

1.4.2.3 Real-Time Quantitative PCR The real-time quantitative PCR reaction mixture (50 L) contained 24 L Hotstart Fluo-PCR mix, 2 L each of forward and reverse primers (25 mol/L), 2 L cDNA, and 20 L ddH O. The PCR conditions were: initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60–63 °C for 30 s (optimized for each primer), and extension at 72 °C for 30 s. All reagents were purchased from Shanghai Bioengineering Co., Ltd. Relative gene expression was calculated using the comparative Ct method, where relative expression = 2^−ΔΔCt = [(Ct_target − Ct_reference)_treatment − (Ct_target − Ct_reference)_control], representing the fold change in target gene expression in the treatment group relative to the control.

1.5 Statistical Analysis

Raw data were organized using Excel 2007 and analyzed using SAS 8.2 software. One-way ANOVA was performed, followed by LSD multiple comparisons. Results are expressed as "mean ± standard error." Differences were considered significant at $P<0.05$.

2.1 Effects of Dietary CP Level on Hepatic Amino Acid Metabolic Enzyme Activities

As shown in Figure 1 [Figure 1: see original paper], on day 10, hepatic GOT activity in the 14% and 17% CP groups was significantly lower than in the control group ($P<0.05$). On day 25, hepatic GOT activity in the 14% CP group was significantly lower than in the other two groups ($P<0.05$). Hepatic GPT activity in the 14% and 17% CP groups was significantly lower than in the control group on day 25 ($P<0.05$), and on day 45, GPT activity in the 14% CP group was significantly lower than in the other two groups ($P<0.05$). Hepatic GS activity in the 14% CP group was significantly lower than in the other two

groups on days 10 and 45 ($P<0.05$), and significantly lower than the control group on day 25 ($P<0.05$). On day 25, hepatic GDH activity in the 14% and 17% CP groups was significantly lower than in the control group ($P<0.05$).

2.2.1 Analysis of qPCR Amplification and Melting Curves

The amplification curves in Figure 2 [Figure 2: see original paper] show stable baselines for GAPDH, SLC6A15, SLC6A20, SLC36A1, and SLC38A2, indicating minimal interfering signals. The no-template control (NTC) remained flat throughout amplification, confirming absence of contamination and primer dimers. Similarly, Figure 3 [Figure 3: see original paper] demonstrates stable baselines during amplification. The melting curves for GAPDH, SLC6A15, SLC6A20, SLC36A1, and SLC38A2 each displayed a single sharp peak, confirming high primer specificity and absence of non-specific products.

2.2.2 Effects of Dietary CP Level on Hepatic Amino Acid Transporter mRNA Expression

As shown in Table 3 , on day 25, the mRNA relative expression of SLC6A15 in the 14% CP group was significantly lower than in the other two groups ($P<0.05$). The mRNA relative expression of SLC36A1 in the 14% and 17% CP groups was significantly lower than in the control group on day 25 ($P<0.05$), and SLC38A2 expression in the 14% CP group was significantly lower than in the control group ($P<0.05$). On day 45, the mRNA relative expression levels of SLC6A20 and SLC38A2 in the 14% and 17% CP groups were significantly lower than in the control group ($P<0.05$), while SLC6A15 expression in the 14% CP group was significantly lower than in the other two groups ($P<0.05$).

3.1 Effects of Dietary CP Level on Hepatic Amino Acid Metabolic Enzyme Activities

The results demonstrate that hepatic GOT and GPT activities in weaned piglets increased with dietary CP level. GOT, also known as aspartate aminotransferase, catalyzes the conversion of -ketoglutarate (-KG) and aspartate (Asp) to glutamate (Glu) and oxaloacetate. GPT, or alanine aminotransferase, catalyzes the conversion of -KG and alanine (Ala) to Glu and pyruvate. Both GOT and GPT are important enzymes in amino acid metabolism and are highly active in the liver. Primarily intracellular, GOT and GPT are released into the bloodstream when tissue cells are damaged, making them important indicators of liver function [5]. Reports on the effects of dietary CP level on porcine hepatic GOT and GPT activities have been inconsistent. Luo [6] reported that insufficient protein nutrition impairs hepatic protein synthesis capacity, leading

to significantly increased plasma GOT and GPT activities. Luo [7] found that plasma GOT and GPT activities in piglets increased initially, then decreased, then increased again with increasing dietary CP level. These findings suggest that the effects of CP level on GOT and GPT activities depend on the nutritional status of the diet (normal, over-nutrition, or deficiency), and that the impact of reducing or increasing CP by the same level differs under different conditions. The present study indicates that reducing dietary CP level may decrease Glu synthesis in piglet liver.

This study also showed that reducing dietary CP level decreased hepatic GS and GDH activities. GS is a key enzyme in ammonia metabolism, present in all organisms, that catalyzes the conversion of L-Glu to glutamine (Gln) [8]. GS plays important roles in nitrogen transport between tissues, detoxification of high ammonia concentrations, and maintenance of acid-base balance. GDH is widely distributed in liver tissue and is crucial for oxidative deamination of amino acids. Typically, GDH catalyzes the synthesis of Glu from -iminoglutarate. Additionally, during amino acid deamination, GDH can work with transaminases to form Gln and asparagine, which are converted to urea [9]. Luo [6] reported that different dietary amino acid compositions had no significant effect on porcine plasma GDH activity. Combined with our findings that reduced dietary CP level decreased hepatic GDH and GS activities, we hypothesize that dietary amino acid quantity is important for hepatic GDH and GS activity.

3.2 Effects of Dietary CP Level on Hepatic Amino Acid Transporter mRNA Expression

The results show that mRNA relative expression of all four detected amino acid transporters decreased to varying degrees with reduced dietary CP level. SLC6A15 is a member of the solute carrier family 6 (SLC6) and functions as a Na - and Cl -dependent neutral amino acid transporter. First identified by Uhl et al. [10] in 1992, it was named SLC6A15, BoAT2, SBAT1, or V7-3 based on its SLC6 function. Takanaga et al. [11] confirmed that SLC6A15 regulates the metabolic conversion of proline (Pro), Met, leucine (Leu), valine (Val), and isoleucine (Ile). Hägglund et al. [12] found that SLC6A15 can alter Leu concentration to regulate energy metabolism in body organs. Drgonova et al. [13] reported that knockout of SLC6A15 in mice decreased Leu and Pro uptake by 40% and 15%, respectively. Hägglund et al. [14] found that SLC6A15 mRNA is primarily expressed in the brain, with detectable expression also in muscle, intestine, liver, and eye.

SLC6A20, also known as SIT1, is a Na - and Cl -dependent amino acid transporter mainly distributed in the intestine and kidney of mammals. It is an important component of Pro metabolism and affects glucose and energy homeostasis by binding Glu, arginine (Arg), and other amino acids [15-16]. Research has shown that SLC6A20 regulates type II diabetes in the kidney [17], and

iminoglycinuria is associated with SLC6A20 mutations [18]. Due to its relatively recent discovery, few reports exist on its regulatory mechanisms in the liver.

SLC36A1, also known as PAT1, encodes a proton-coupled amino acid transporter primarily distributed in the intestine and kidney, with low mRNA expression in the liver [15]. SLC36A1 transports small amino acids such as Ala, Pro, and glycine (Gly). Under certain H concentrations, SLC36A1 selectively activates the Na /H exchanger III to generate imino acids, thereby maintaining normal H concentration in the body [19]. Chen et al. [20] found SLC36A1 expression in human small intestine, brain, liver, testis, and kidney tissues.

SLC38A2 is a Na -dependent neutral amino acid transporter belonging to the SLC6 family, expressed in nearly all mammalian tissues [21]. Ortiz et al. [22] found that feeding rats a high-protein diet increased SLC38A2 expression, suggesting that SLC38A2 oxidizes and degrades excess protein in the form of amino acids. Conti et al. [23] reported that SLC38A2 binds Gln for cellular uptake and participates in the Glu-Gln metabolic cycle. Our study found that hepatic SLC38A2 mRNA relative expression in weaned piglets increased with dietary CP level, consistent with the findings of Ortiz et al. [22].

The trend of hepatic amino acid transporter mRNA expression in response to dietary CP level was consistent with that of amino acid metabolic enzyme activities. Overall, reducing dietary CP level by 3% and 6% decreased the quantity of certain amino acids (such as Glu, Pro, Arg, Leu, etc.) entering hepatocytes, thereby reducing the metabolic rate of these amino acids in the liver.

Conclusions

1. Reducing dietary CP level by 3% and 6% significantly decreased the activities of hepatic amino acid metabolic enzymes (GOT, GPT, GS, GDH) in weaned piglets.
2. Reducing dietary CP level by 3% and 6% significantly decreased the mRNA relative expression levels of hepatic amino acid transporters (SLC6A15, SLC6A20, SLC36A1, SLC38A2) in weaned piglets.

References

[1] KERR B J, EASTER R A. Effect of feeding reduced protein, amino acid-supplemented diets on nitrogen and energy balance in grower pigs[J]. Journal of Animal Science, 1995, 73(10): 3000–3008.

[2] LE BELLEGO L, VAN MILGEN J, NOBLET J. Effect of high temperature and low-protein diets on performance of growing-finishing pigs[J]. Journal of Animal Science, 2002, 80(3): 691–701.

[3] CHEN C. Study on the effects of dietary protein level on amino acid metabolism and conversion in piglet liver[D]. Master' s thesis. Chongqing: Southwest University, 2015: 21-24.

[4] ZHANG L Y. Feed Analysis and Feed Quality Detection Technology[M]. 3rd ed. Beijing: China Agricultural University Press, 2007.

[5] ZOU S X. Animal Biochemistry[M]. 5th ed. Beijing: China Agricultural Press, 2008.

[6] LUO J Q. Comparative study on metabolic effects of different protein sources in pig diets[D]. PhD thesis. Ya' an: Sichuan Agricultural University, 2011.

[7] LUO H M. Effects of different dietary protein levels on growth performance, blood biochemical indices, and immune function in early-weaned piglets[D]. Master' s thesis. Sichuan: Sichuan Agricultural University, 2005.

[8] SHAKED I, BEN-DROR I, VARDIMON L. Glutamine synthetase enhances the clearance of extracellular glutamate by the neural retina[J]. Journal of Neurochemistry, 2002, 83(3): 574-580.

[9] LI Z Q, WEI M J. Detection and clinical significance of serum glutamate dehydrogenase[J]. Foreign Medical Sciences (Section of Clinical Biochemistry and Laboratory Medicine), 2001, 22(4): 209-210.

[10] UHL G R, KITAYAMA S, GREGOR P, et al. Neurotransmitter transporter family cDNAs in a rat midbrain library: 'orphan transporters' suggest sizable structural variations[J]. Molecular Brain Research, 1992, 16(3/4): 353-359.

[11] TAKANAGA H, MACKENZIE B, PENG J B, et al. Characterization of a branched-chain amino-acid transporter SBAT1 (SLC6A15) that is expressed in human brain[J]. Biochemical and Biophysical Research Communications, 2005, 337(3): 892-900.

[12] HÄGGLUND M G A, ROSHANBIN S, LÖFQVIST E, et al. B0AT2 (SLC6A15) is localized to neurons and astrocytes, and is involved in mediating the effect of leucine in the brain[J]. PLoS One, 2013, 8(3): 58651.

[13] DRGONOVA J, LIU Q R, HALL F S, et al. Deletion of v7-3 (SLC6A15) transporter allows assessment of its roles in synaptosomal proline uptake, leucine uptake and behaviors[J]. Brain Research, 2007, 1183: 10-20.

[14] HÄGGLUND M G A, HELLSTEN S V, BAGCHI S B, et al. Characterization of the transporter B0AT3 (SLC6A17) in rodent central nervous system[J]. BMC Neuroscience, 2013, 14: 54.

[15] TAKANAGA H, MACKENZIE B, SUZUKI Y, et al. Identification of mammalian proline transporter SIT1 (SLC6A20) with characteristics of classical system imino[J]. The Journal of Biological Chemistry, 2005, 280(10): 8974-8984.

[16] JOBGEN W S, FRIED S K, FU W J, et al. Regulatory role for the arginine-nitric oxide pathway in metabolism of energy substrates[J]. Journal of Nutri-

tional Biochemistry, 2006, 17(9): 571-588.

[17] PATTERSON A D, BONZO J A, LI F, et al. Metabolomics reveals attenuation of the SLC6A20 kidney transporter in nonhuman primate and mouse models of type 2 diabetes mellitus[J]. Journal of Biological Chemistry, 2011, 286(22): 19511-19522.

[18] BRÖER S, BAILEY C G, KOWALCZUK S, et al. Iminoglycinuria and hyperglycinuria are discrete human phenotypes resulting from complex mutations in proline and glycine transporters[J]. Journal of Clinical Investigation, 2008, 118(12): 3881-3892.

[19] ANDERSON C M H, THWAITES D T. Indirect regulation of the intestinal H+-coupled amino acid transporter hPAT1 (SLC36A1)[J]. Journal of Cellular Physiology, 2005, 204(2): 604-613.

[20] CHEN P E, GEBALLE M T, STANSFELD P J, et al. Structural features of the glutamate binding site in recombinant NR1/NR2A N-methyl-D-aspartate receptors determined by site-directed mutagenesis and molecular modeling[J]. Molecular Pharmacology, 2005, 67(5): 1470-1484.

[21] YAO D D, MACKENZIE B, MING H, et al. A novel system A isoform mediating Na+/neutral amino acid cotransport[J]. The Journal of Biological Chemistry, 2000, 275(30): 22790-22797.

[22] ORTIZ V, ALEM N G, ESCAMILLA-DEL-ARENAL M, et al. Promoter characterization and role of CRE in the basal transcription of the rat SNAT2 gene[J]. American Journal of Physiology, 2011, 300(6): E1092-E1102.

[23] CONTI F, MELONE M. The glutamine commute: lost in the tube?[J]. Neurochemistry International, 2006, 48(6/7): 459-464.

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