Effects of Early Weaning on Excitatory Amino Acid Transporter 1 Expression in the Jejunum and Ileum of Piglets: Postprint

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Abstract

The present experiment was designed to investigate differences in small intestinal glutamate transporter gene expression between early-weaned piglets and suckling piglets during the 10–20 day period postpartum. Forty 10-day-old "Duroc × Landrace × Large White" crossbred piglets with similar body weights were selected from 40 litters of different sows and randomly divided into two groups of 20 piglets each. The control group (suckling group) remained with their dams, while the experimental group (weaning group) was weaned and raised in isolation; the experimental period lasted 10 days. At the end of the feeding period, 12 piglets were randomly selected from each group, slaughtered, and the jejunum and ileum were collected to determine protein expression of the glutamate transporter excitatory amino acid transporter 1 (EAAC1) and free amino acid content. Results showed that weaning significantly decreased protein and gene expression levels of EAAC1 (57 and 73 ku) and its associated protein glutamate transporter-associated protein (GTRAP3-18) (50 ku) in the jejunum and ileum of piglets ($P<0.05$). Weaning increased free glutamate and total amino acid contents in the jejunum but decreased them in the ileum, with significant differences ($P<0.05$). These results suggest that early weaning reduces the protein content of EAAC1 and GTRAP3-18, which may be related to impaired intestinal amino acid absorption and transport caused by nutritional glutamate deficiency in early-weaned piglets.

Full Text

Effects of Early Weaning on Jejunal and Ileal Excitatory Amino Acid Carrier 1 Expression in Piglets

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Abstract: This study investigated the differential expression of intestinal glutamate transporter genes between early-weaned and suckling piglets from 10 to 20 days of age. Forty "Duroc × Landrace × Yorkshire" crossbred piglets with similar body weight were selected from 40 different sows at 10 days of age and randomly divided into two groups (n=20 each). The control group (suckling group) remained with their dams, while the experimental group (weaned group) was weaned and housed separately. The trial lasted 10 days. At the end of the experiment, 12 piglets were randomly selected from each group and euthanized to collect jejunal and ileal tissues for determination of excitatory amino acid carrier 1 (EAAC1) protein expression and free amino acid content. The results showed that weaning significantly reduced EAAC1 (57 and 73 ku) and its associated protein glutamate transporter associate protein 3-18 (GTRAP3-18) (50 ku) protein and gene expression in both jejunum and ileum ($P<0.05$). Weaning increased free glutamate and total amino acid contents in jejunum but decreased them in ileum, with significant differences ($P<0.05$). These findings suggest that early weaning reduces EAAC1 and GTRAP3-18 protein levels, which may be associated with impaired intestinal amino acid absorption and transport caused by nutritional glutamate deficiency in early-weaned piglets.

Keywords: piglet; weaning; glutamate transporter; EAAC1; small intestine

Introduction

Glutamate (Glu) serves as one of the primary energy substrates for animal mucosa and is a conditionally essential amino acid during piglet weaning, playing a critical role in piglet growth, development, and intestinal mucosal growth and repair. Glutamate cannot be passively absorbed in the intestine and must be actively transported via specific carrier proteins. The excitatory amino acid transporter (EAAT) family represents sodium-dependent, high-affinity glutamate transporters that maintain glutamate homeostasis in the nervous system. Among these, excitatory amino acid carrier 1 (EAAC1) is the most important member, not only because its glutamate transport rate in the nervous system is nearly 10 times faster than other transporters, but also because it is not nervous system-specific and is expressed in non-neural tissues such as the small intestine. EAAC1 expression is negatively regulated by glutamate transporter associate protein 3-18 (GTRAP3-18). Studies in human cancer and epilepsy have shown that GTRAP3-18 inhibits EAAC1-mediated glutamate transport by binding to the C-terminus of EAAC1, causing glutamate toxicity in the nervous system.

Notably, GTRAP3-18 exclusively binds to and regulates EAAC1 without interacting with other EAAT family members, and EAAC1 expression is modulated by GTRAP3-18 expression levels.

While most EAAC1 research has focused on the nervous system, few studies have examined its role in the intestine. EAAC1 knockout mice exhibit age-dependent loss of substantia nigra dopaminergic neurons and increased oxidative stress, and abnormal EAAC1 accumulation has been observed in hippocampal neurons of Alzheimer' s disease patients. Fu et al. (2012) first cloned EAAC1 from the jejunum of suckling piglets and demonstrated that its expression varies during lactation, with low expression in low birth weight piglets. These findings collectively indicate that EAAC1 expression correlates with abnormal development, stress, and disease states. Low EAAC1 expression impairs glutamate transport efficiency, subsequently affecting small intestinal mucosal development and absorptive function in piglets. Weaning represents a physiological stressor that eliminates maternal glutamate intake, yet how EAAC1 expression changes in the small intestine during this process remains unknown. Building upon previous research, this study used suckling piglets as controls to investigate changes in EAAC1 and its regulatory protein GTRAP3-18 expression in the jejunum and ileum during weaning, aiming to elucidate the impact of weaning stress on EAAC1 expression and provide a theoretical basis for understanding intestinal mucosal damage repair mechanisms and improving growth performance during the weaning transition.

Materials and Methods

1.1 Animal Grouping and Management The experiment was conducted in May 2010 at a pig production demonstration base in Qiqihar, Heilongjiang Province. Forty "Duroc × Landrace × Yorkshire" crossbred piglets at 10 days of age with an average body weight of (4.48$±$0.26) kg were selected from 40 different sows and randomly divided into two groups using a single-factor experimental design, with 20 replicates per group (one piglet per replicate). The trial lasted 10 days.

Suckling group piglets remained with their dams in the nursing facility for 10 days. Weaned group piglets were housed in a nursery facility and fed a corn-soybean meal-based commercial weaning diet for 10 days, with three daily feedings (morning, noon, and evening) and ad libitum access to water. The basal diet composition and nutrient levels are presented in .

The nursery facility was enclosed with good ventilation and concrete floors. Piglets were individually housed in single pens, with an infrared heat lamp suspended 0.8 m above ground level in one corner of each pen. A 0.5 m × 1.0 m heat-absorbing pad was placed beneath the lamp to ensure piglet warmth.

1.2 Sample Collection and Processing On the morning of day 11, 12 piglets were randomly selected from each group (24 total). The abdominal cavity was opened to excise the jejunum and ileum, which were immediately flushed with ice-cold physiological saline. Samples were taken from the middle segment of each intestinal section and snap-frozen in liquid nitrogen. Frozen intestinal samples were ground into powder using a mortar under liquid nitrogen and stored at -80 °C.

1.3 Sample Preparation Tissue Homogenization: Approximately 1.3 g of powdered frozen intestinal tissue was weighed and thawed in ice-cold homogenization buffer containing protease inhibitors at a ratio of 1 g tissue to 20 mL buffer. Samples were homogenized using a multi-layer homogenizer at 16,000 r/min for 3 minutes, with a 20-second pause every minute. The total volume of homogenate was recorded, and 2 mL aliquots were stored at -80 °C.

Intracellular Fraction: Intracellular fraction samples were prepared using magnesium precipitation and differential centrifugation at 4 °C.

Apical Membrane: The remaining intracellular fraction samples were further processed via differential centrifugation to prepare apical membrane samples.

1.4.1 Protein Expression Quantification Protein Content Determination: Bovine serum albumin (fraction V) was used as the protein standard to determine protein concentrations in tissue homogenates, intracellular fractions, and apical membrane samples.

Western Blot Analysis: Samples were prepared at a concentration of 1 µg/µL. Western blot was performed using β-actin as a housekeeping control. Primary antibodies included: goat anti-human EAAC1 polyclonal antibody (Sc-7761, Santa Cruz Biotechnology) at 1:2,000 dilution; mouse anti-human GTRAP3-18 polyclonal antibody (H00010550-A01, Abnova) at 1:2,000 dilution; and mouse anti-human β-actin monoclonal antibody (Bio-Rad) at 1:10,000 dilution. The secondary antibody was rabbit anti-human IgG (Bio-Rad) at 1:10,000 dilution.

1.4.2 Gene Expression Quantification Oligonucleotide Primer Design: Primer 5.0 software was used to design primers for target and housekeeping genes based on GenBank cDNA sequences (), which were synthesized by Invitrogen. To avoid contamination from non-specific genomic DNA, all piglet mRNA sequences were aligned with corresponding porcine gene sequences using Spidey software, ensuring all primers spanned at least two exon regions.

RNA Preparation: Total RNA was extracted from tissue samples using TRIzol reagent (Invitrogen), treated with DNase (Invitrogen), and reverse-transcribed into cDNA using the iScript cDNA synthesis kit according to manufacturer instructions.

Real-Time Quantitative RT-PCR: A 25 µL reaction system was used following the iQ SYBR Green Supermix RT-PCR kit (Qiagen) protocol. The

RT-PCR program consisted of: reverse transcription (50 °C, 30 min), protein denaturation (95 °C, 15 min), amplification and quantification for 45 cycles (95 °C denaturation for 15 s, 54 °C annealing for 15 s, 72 °C extension for 15 s), and melting curve analysis (heating from 60 to 99 °C at 0.1 °C/s with fluorescence measurement).

Data Calculation: The relative expression ratio of target gene to housekeeping gene was calculated using the formula: R = 2^(-ΔCt), where R represents the relative expression ratio and Ct represents the threshold cycle number. Both target and housekeeping genes were amplified above 30 fluorescence units. Optimal RT-PCR efficiency was determined from serial RNA dilutions using the formula 10^(-1/slope), with consistent values obtained for both target genes and β-actin.

1.5 Determination of Free Amino Acid Content in Piglet Small Intestine Free amino acid content was determined using high-performance liquid chromatography equipped with a binary solvent system, automatic sampler, data workstation, and fluorescence detector.

1.6 Statistical Analysis Protein expression levels were expressed as relative protein abundance. Western blot bands were scanned using Quantity One software (Bio-Rad) and converted to density values, with target protein/β-actin density ratios calculated as relative protein content.

Data were analyzed using one-way ANOVA in SAS 9.0 software. Results are expressed as means ± SEM or pooled SEM. Differences were considered significant at $P<0.05$. Graphs were generated using Fig.P curve fitting software.

Results and Analysis

2.1 Effects of Weaning on Piglet Growth Performance As shown in , during the 10-day trial, weaned piglets had an average daily feed intake of $(148.50\$\pm16.90)g/dandafeed-to-gainratioof3.58\pm\$2.34$. Compared with suckling piglets, weaned piglets showed significantly lower final body weight ($P<0.05$) and extremely significantly lower average daily gain ($P<0.01$). Early weaning significantly impacted piglet growth performance.

2.2.1 Effects of Early Weaning on EAAC1 Protein Expression in Piglet Small Intestine Western blot analysis successfully detected EAAC1 protein in tissue homogenates, intracellular fractions, and apical membranes of piglet small intestine. EAAC1 protein exhibited a molecular weight of 57 ku in tissue homogenates and intracellular fractions, and 73 ku in apical membranes. Using β-actin as a reference, EAAC1 protein abundance in weaned piglets decreased by 25%, 21%, and 9% in jejunal tissue homogenates, intracellular fractions, and apical membranes, respectively, compared with suckling

piglets (P<0.05) ([Figure 1: see original paper]). In ileal tissue homogenates, intracellular fractions, and apical membranes, EAAC1 protein abundance decreased by 32%, 22%, and 14%, respectively (P<0.05) ([Figure 2: see original paper]). These results demonstrate that EAAC1 protein is present in piglet small intestine and its abundance is reduced by early weaning.

2.2.2 Effects of Early Weaning on EAAC1 mRNA Expression in Piglet Small Intestine EAAC1 mRNA expression analysis revealed that, relative to β-actin, EAAC1 mRNA expression in weaned piglets decreased by 88% in jejunum and 73% in ileum compared with suckling piglets (P<0.05) ().

Pearson correlation analysis showed positive linear relationships between EAAC1 protein abundance and EAAC1 mRNA expression in jejunal tissue homogenates (r=0.52, P=0.042, n=24), intracellular fractions (r=0.56, P=0.021, n=24), and apical membranes (r=0.49, P=0.008, n=24) across both groups (P<0.05). Significant positive linear relationships were also observed between EAAC1 protein levels in jejunal tissue homogenates and intracellular fractions, and between intracellular and apical membrane fractions (P<0.05). Similarly, in ileum, positive linear relationships existed between EAAC1 protein abundance and mRNA expression in tissue homogenates (r=0.51, P=0.021, n=24), intracellular fractions (r=0.51, P=0.016, n=24), and apical membranes (r=0.41, P=0.016, n=24), with significant positive correlations between protein levels across fractions (P<0.05).

2.3.1 Effects of Early Weaning on GTRAP3-18 Protein Expression in Piglet Small Intestine GTRAP3-18 protein with a molecular weight of 50 ku was detected in jejunal and ileal tissue homogenates, intracellular fractions, and apical membranes ([Figure 3: see original paper], [Figure 4: see original paper]). Using β-actin as a reference, GTRAP3-18 protein abundance in weaned piglets decreased by 15%, 28%, and 55% in jejunal tissue homogenates, intracellular fractions, and apical membranes, respectively (P<0.05). In ileum, GTRAP3-18 protein abundance decreased by 16%, 7%, and 27% in tissue homogenates, intracellular fractions, and apical membranes, respectively (P<0.05).

2.3.2 Effects of Early Weaning on GTRAP3-18 mRNA Expression in Piglet Small Intestine GTRAP3-18 mRNA expression analysis showed that, relative to β-actin, expression decreased by 70% in jejunum and 52% in ileum of weaned piglets compared with suckling piglets (P<0.05) ().

Pearson correlation analysis revealed positive linear relationships between GTRAP3-18 protein expression and mRNA levels in jejunal tissue homogenates (r=0.33, P=0.027, n=24), intracellular fractions (r=0.54, P=0.019, n=24), and apical membranes (r=0.56, P=0.028, n=24) (P<0.05). Significant positive correlations were also observed between GTRAP3-18 protein levels across jejunal fractions. In ileum, positive linear relationships existed between GTRAP3-18 protein expression and mRNA levels in tissue homogenates

(r=0.42, P=0.014, n=24), intracellular fractions (r=0.42, P=0.047, n=24), and apical membranes (r=0.15, P=0.029, n=24), with significant positive correlations between protein levels across fractions (P<0.05).

2.4.1 Effects of Early Weaning on Free Amino Acid Content in Jejunum Compared with suckling piglets, the substrate amino acid for EAAC1, glutamate, increased by 26% in jejunal contents of weaned piglets (P<0.05). Glutamine, the substrate for ASC amino acid transporter 2 (ASCT2), showed no significant change (P>0.05). Other free amino acids including threonine, glycine, and ornithine increased by 57%, 88%, and 53%, respectively (P<0.05) ([Figure 5: see original paper]).

2.4.2 Effects of Early Weaning on Free Amino Acid Content in Ileum In contrast to jejunal changes, weaned piglets showed opposite trends in ileal free amino acid content. Glutamate, the substrate for EAAC1, decreased by 43% compared with suckling piglets (P<0.05). Glutamine, the substrate for ASCT2, decreased by 52% (P<0.05). Other amino acids including lysine, methionine, phenylalanine, tryptophan, arginine, taurine, and tyrosine all decreased by more than 42% (P<0.05) ([Figure 6: see original paper]).

Discussion

Weaning induces complex morphological and functional changes involving nutrition, digestion and metabolism, stress, neuroendocrinology, and gene function. Post-weaning daily gain is a crucial indicator of piglet growth. Compared with suckling piglets, unaffected daily gain would indicate adequate dietary nutrition and successful adaptation from milk to feed, with concurrent intestinal development. However, in practice, 10% of piglets die from this physiological stress, resulting from both environmental separation from the sow and intestinal stress from the dietary transition. Weaning stress not only alters gastrointestinal function but also affects brain-gut axis regulation. The nutritional and environmental changes during weaning reduce feed intake, and due to immature gastrointestinal development, weaning impairs piglet growth performance. In this study, early-weaned piglets exhibited clear weaning stress symptoms, with average daily feed intake of only 148.5 g—substantially below the 240 g/d recommended by NRC (1998). Average daily gain was significantly lower than in suckling piglets. Gu et al. reported that weaning stress causes substantial changes in intestinal structure and function, directly affecting digestive and absorptive capacity and consequently growth performance.

EAAC1 is the primary glutamate transporter, widely distributed throughout the cell body. Most research has focused on EAAC1-mediated glutamate transport in the central nervous system, with few studies examining its expression in porcine intestinal mucosa. This study investigated changes in EAAC1 protein expression and mRNA levels to reveal weaning effects on glutamate transport.

Results demonstrated that early weaning significantly reduced both EAAC1 protein and mRNA expression in piglet small intestine. Watabe et al. demonstrated in HEK293 cells that GTRAP3-18 negatively regulates EAAC1 activity and intracellular glutathione content, thereby affecting susceptibility to oxidative stress. Differentiation, heat stress, and oxidative stress increase GTRAP3-18 protein dissociation in humans, suggesting that GTRAP3-18 expression should increase rather than decrease during oxidative stress. Our findings showed that early weaning reduced both GTRAP3-18 protein and mRNA expression in piglet jejunum and ileum, indicating that weaning stress produces different responses compared with heat stress and other oxidative stressors. Weaned piglets experiencing physiological stress require increased dietary glutamate for intestinal mucosal protection. Studies have shown that increased glutamate uptake efficiency reduces GTRAP3-18 protein expression. Our results demonstrated elevated jejunal glutamate concentration accompanied by reduced GTRAP3-18 protein expression in weaned piglets, consistent with Lin et al. However, ileal glutamate content decreased despite reduced GTRAP3-18 expression, suggesting that the specific role and mechanism of GTRAP3-18 under stress or disease conditions require further investigation.

As an excitatory amino acid transported by EAAC1, glutamate showed contrasting patterns between intestinal segments in weaned piglets. Ileal free glutamate decreased significantly while jejunal content increased. Previous studies indicate that EAAC1 expression is predominant in jejunum, and glutamate transmembrane transport efficiency is regulated by EAAC1 expression changes. We hypothesize that abundant EAAC1 in jejunum transports large amounts of free glutamate to enhance uptake, leading to EAAC1 consumption and reduced expression. Conversely, lower EAAC1 expression in ileum results in reduced transport efficiency, causing decreased ileal glutamate content compared with suckling piglets. However, glutamate content is influenced not only by transport but also by the glutamate-glutamine cycle.

In summary, early weaning stress reduces apical membrane EAAC1 expression in jejunum. The consumed EAAC1 enhances glutamate transport, increasing jejunal free glutamate and other amino acids while decreasing GTRAP3-18 expression. In contrast, reduced apical membrane EAAC1 expression in ileum decreases glutamate transport efficiency and capacity, resulting in reduced ileal glutamate and other free amino acid contents.

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