Effects of nutrition, Escherichia coli, and amino acids on antimicrobial peptide and signaling pathway protein expression in porcine small intestinal epithelial cells: Postprint

Authors: Ren Man, Gong Bishuang, Jin Erhui, Li Shenghe, Zeng Xiangfang, Qiao Shiyan

Date: 2017-10-10T00:00:00+00:00

Abstract

To investigate the effects and molecular mechanisms of stress and amino acids on antimicrobial peptide expression in epithelial cells, the study utilized the porcine small intestinal epithelial cell line IPEC-J2 as the experimental model, with starvation and Escherichia coli infection serving as nutritional and bacterial stressors, respectively, and alanine and isoleucine administered as amino acid treatments. Cellular mRNA was collected, and quantitative real-time PCR was employed to determine the expression levels of β-defensins and related signaling pathway proteins. The results demonstrated that, compared with the control group (DMEM/F12 medium), starvation significantly reduced the expression of porcine β-defensin 2 (pBD-2), porcine β-defensin 3 (pBD-3), porcine β-defensin EP2c (pEP2c), silent information regulator 2 homolog 1 (Sirt1), forkhead transcription factor 1 (FoxO1), and forkhead transcription factor 4 (FoxO4) in small intestinal epithelial cells ($P<0.05$), whereas Escherichia coli infection had no significant effect on β-defensin expression ($P>0.05$). Compared with the control group (starvation medium), alanine treatment did not significantly affect the expression of pBD-2 and pBD-3 ($P>0.05$), but significantly elevated pEP2c expression levels ($P<0.05$); isoleucine treatment significantly increased the expression levels of pBD-2, pBD-3, and pEP2c ($P<0.05$). Compared with the control group, alanine and isoleucine treatments differentially affected the transcription of signaling pathway proteins, with alanine treatment significantly enhancing the expression levels of Sirt1 and FoxO4 ($P<0.05$), and isoleucine treatment significantly promoting the expression of Sirt1, FoxO1, and FoxO4 ($P<0.05$). It was concluded that nutritional stress decreases β-defensin expression in porcine small intestinal epithelial cells, whereas amino acid supplementation can promote their expression, and this regulatory effect may be associated with Sirt1

and forkhead transcription factor (FoxO) signaling pathway proteins.

Full Text

Effects of Nutrition, *Escherichia coli*, and Amino Acids on Expression of Antimicrobial Peptides and Signaling Pathway Proteins in Porcine Intestinal Epithelial Cells

REN Man[1],[2], GONG Bishuang[1], JIN Erhui[1], LI Shenghe[1], ZENG Xiangfang[2], QIAO Shiyan[2]*

[1]College of Animal Science, Anhui Science and Technology University, Fengyang 233100, China
[2]College of Animal Science and Technology, China Agricultural University, Beijing 100193, China

Abstract

This study investigated the effects and molecular mechanisms of stress and amino acids on antimicrobial peptide expression in epithelial cells using the porcine intestinal epithelial cell line IPEC-J2 as a model. Starvation and *Escherichia coli* infection were employed as nutritional and bacterial stressors, respectively, while alanine and isoleucine served as amino acid treatments. Cell mRNA was collected, and expression levels of β-defensins and related signaling pathway proteins were measured using real-time quantitative PCR.

The results demonstrated that compared with the control group (DMEM/F12 medium), starvation significantly reduced the expression of porcine β-defensin 2 (pBD-2), porcine β-defensin 3 (pBD-3), porcine β-defensin EP2c (pEP2c), silent mating type information regulation 2 homolog 1 (Sirt1), forkhead transcription factor 1 (FoxO1), and forkhead transcription factor 4 (FoxO4) ($P<0.05$). In contrast, *E. coli* treatment did not significantly affect β-defensin expression ($P>0.05$).

Compared with the starvation control group, alanine treatment did not significantly influence pBD-2 or pBD-3 expression ($P>0.05$) but significantly increased pEP2c expression levels ($P<0.05$). Isoleucine treatment significantly elevated the expression of all three defensins: pBD-2, pBD-3, and pEP2c ($P<0.05$). Both amino acid treatments differentially affected signaling pathway protein transcription. Alanine significantly increased Sirt1 and FoxO4 expression ($P<0.05$), while isoleucine significantly promoted expression of Sirt1, FoxO1, and FoxO4 ($P<0.05$).

These findings indicate that nutritional stress decreases β-defensin expression in porcine intestinal epithelial cells, whereas amino acid supplementation enhances

their expression. This regulatory effect appears to be mediated through Sirt1 and forkhead transcription factor (FoxO) signaling pathway proteins.

Keywords: porcine intestinal epithelial cell; antimicrobial peptide; defensin; signaling pathway; Sirt1; FoxO

Introduction

Antibiotics have been used in livestock production for over 50 years to reduce disease incidence, improve growth rates, and enhance feed utilization. However, antibiotic misuse has led to the development of antimicrobial resistance in numerous human and animal pathogens, posing a direct threat to global public health. Research has shown that even short-term, low-dose antibiotic use can increase the quantity and diversity of antimicrobial resistance genes in the porcine gut microbiome. Consequently, developing strategies to enhance animal immunity and disease resistance has become urgent. Recent findings suggest that nutritional modulation of innate immune function in the intestinal tract represents a viable approach.

Endogenous antimicrobial peptides (AMPs) synthesized by mammals exhibit broad-spectrum antimicrobial activity, participate in innate immune defense, and are less likely to induce microbial resistance. Intestinal epithelial cells secrete diverse AMPs to protect against complex microbial environments and pathogen invasion, forming an innate immune barrier alongside secretory immunoglobulin A (sIgA) on the mucosal surface. Growing evidence indicates that AMPs also function as immune regulators by modulating chemotaxis and Toll-like receptor (TLR) signaling intensity. To date, 25 AMPs have been identified in pigs, including 11 cathelicidins, 12 defensins, 1 saposin, and 1 cecropin. Multiple factors influence endogenous AMP expression, including developmental stage, injury, nutrients (such as vitamin D, short-chain fatty acids, and amino acids), and lipopolysaccharide (LPS). Previous studies demonstrated that isoleucine modulates intestinal mucosal immune function in weaned piglets and promotes AMP expression. However, limited research exists on how nutritional levels affect AMP expression in porcine intestinal epithelial cells, and the molecular mechanisms underlying AMP induction remain poorly understood. Therefore, this study employed the porcine intestinal epithelial cell line IPEC-J2 as a model to investigate the effects of nutritional and bacterial stress, along with different amino acid treatments, on AMP and signaling pathway protein expression. The findings aim to provide insights into stress and nutrient regulation of endogenous AMP expression and intestinal barrier function, and to establish a theoretical basis for screening nutrients with immune-enhancing properties.

1.1 Cell Culture

The porcine intestinal epithelial cell line IPEC-J2, generously provided by Professor Guoyao Wu (Texas A&M University), was maintained in our laboratory. This cell line was originally isolated from the jejunal epithelium of newborn piglets and grows as a monolayer. The culture medium consisted of DMEM/F12 (Hyclone, China) supplemented with 5% fetal bovine serum (FBS) (Gibco, USA), 1% insulin-transferrin-selenium (ITS) (ScienCell, USA), and 1 g/L endothelial growth factor (EGF) (Sigma, USA).

1.2 Experimental Design and Treatments

IPEC-J2 cells were seeded in 6-well plates with complete medium and grown to 80% confluence before treatment. For nutritional and bacterial stress studies, the culture medium was replaced with: (1) control group: DMEM/F12 medium; (2) starvation group: starvation medium (Earle' s balanced salt solution + vitamin mixture); or (3) *E. coli* group: DMEM/F12 medium containing 1$×10^{3}$ CFU/well *E. coli*. Cells were treated for 12 hours before collection.

For amino acid treatments, the culture medium was replaced with: (1) control group: starvation medium; (2) alanine group: starvation medium supplemented with 1.0 mmol/L alanine (isoleucine nitrogen-equivalent group); or (3) isoleucine group: starvation medium supplemented with 1.0 mmol/L isoleucine. Cells were treated for 12 hours before collection.

1.3 Total mRNA Extraction and Reverse Transcription

Total mRNA from intestinal epithelial cells was extracted using RNAzol® RT reagent (Molecular Research Center, USA) according to the manufacturer' s instructions. mRNA concentration and quality were assessed using a Nanodrop spectrophotometer; samples with OD260/OD280 ratios of 1.8–2.0 and OD260/OD230 ratios of 1.5–2.2 were considered high quality and suitable for subsequent analysis. mRNA was reverse-transcribed into cDNA using a reverse transcription kit (TaKaRa, China). The housekeeping gene β-actin was amplified by PCR to verify reverse transcription efficiency. cDNA was stored at –20°C for further use.

1.4 Gene Primer Design

Primers for the reference gene (β-actin), AMP genes [porcine β-defensin 2 (pBD-2), porcine β-defensin 3 (pBD-3), and porcine β-defensin EP2c (pEP2c)], and signaling pathway protein genes [silent mating type information regulation 2 homolog 1 (Sirt1), forkhead transcription factor 1 (FoxO1), and forkhead transcription factor 4 (FoxO4)] were designed using Primer Premier 6.0 software and synthesized by Beijing Sanbo Zhiyuan Biotechnology Co., Ltd. Optimal annealing temperatures and amplification specificity were determined by gradient PCR and agarose gel electrophoresis. Primers without dimers or non-specific amplification were selected for use; sequences are shown in .

1.5 Real-Time Quantitative PCR

Gene fragments were amplified from extracted cDNA using corresponding primers, purified, and serially diluted 10^{-1} to 10^{-9}. Eight dilutions (10^{-2} to 10^{-9}) were used to generate standard curves with three replicates per dilution in preliminary experiments to verify primer amplification efficiency. Reverse-transcribed cDNA served as template for real-time quantitative PCR using SYBR Green PCR reagent (TaKaRa, China) in a 10 L reaction system. For β-actin detection, cDNA amount was 1/20 of the reaction volume; for target genes, cDNA amount was 1/10. PCR was performed on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, USA) with the following program: 95°C for 5 s, 60°C for 34 s, 72°C for 10 s, for 42 cycles.

1.6 Data Analysis

Gene expression was analyzed using the 2ˆ(-ΔCt) method, with relative expression levels calculated using the housekeeping gene as reference. Each treatment had three replicates. Relative expression values were subjected to one-way ANOVA using SAS 8.1 (SAS Institute, Gary, USA). If significant differences were detected among treatments, Duncan' s multiple comparison test was applied. Results are expressed as mean ± standard error, with P<0.05 considered statistically significant.

2.1 Effects of Stress and Amino Acid Treatments on AMP Expression in IPEC-J2 Cells

The porcine intestinal epithelial cell line IPEC-J2 exhibited monolayer growth [Figure 1: see original paper] with rapid proliferation that decreased when confluence exceeded 90%. This study identified three porcine β-defensin genes (pBD-2, pBD-3, and pEP2c) expressed in IPEC-J2 cells for quantitative analysis.

Nutritional and bacterial stress effects on defensin transcription in IPEC-J2 cells are shown in [Figure 2: see original paper]. Compared with the control group, supplementation with 1$×10ˆ{3}$ CFU/well *E. coli* did not significantly affect pBD-2, pBD-3, or pEP2c expression (P>0.05). However, starvation stress significantly reduced defensin expression (P<0.05), decreasing pBD-2 to 0.47-fold, pBD-3 to 0.58-fold, and pEP2c to 0.25-fold of control levels.

Amino acid treatments produced different outcomes. Alanine did not significantly affect pBD-2 or pBD-3 expression (P>0.05) but significantly increased pEP2c expression (P<0.05). Isoleucine significantly enhanced expression of all three defensins (P<0.05), increasing pBD-2 by 5.68-fold, pBD-3 by 9.94-fold, and pEP2c by 5.34-fold compared with the starvation control. Alanine served as a nitrogen-equivalent control for isoleucine to determine whether the observed effects were due to isoleucine itself or simply nitrogen supplementation. The results indicate that while nitrogen supplementation can influence pEP2c

expression, isoleucine exerts a much stronger, nitrogen-independent promoting effect on defensin expression.

2.2 Effects of Nutritional Stress and Amino Acid Treatments on Signaling Pathway Protein Transcription

This study examined three signaling pathway proteins: Sirt1, FoxO1, and FoxO4. Compared with the control group, starvation stress significantly reduced expression of all three proteins in IPEC-J2 cells ($P<0.05$), decreasing Sirt1 to 0.36-fold, FoxO1 to 0.31-fold, and FoxO4 to 0.39-fold of control levels [Figure 3: see original paper].

Both alanine and isoleucine differentially affected signaling pathway protein transcription. Alanine significantly increased Sirt1 (2.13-fold) and FoxO4 (3.16-fold) expression ($P<0.05$). Isoleucine markedly enhanced expression of Sirt1 (30.76-fold), FoxO1 (3.45-fold), and FoxO4 (3.00-fold) ($P<0.05$).

Discussion

The intestinal mucosa faces numerous challenges, including maintaining commensal microbial balance and preventing pathogen invasion. Antimicrobial peptides on the epithelial surface play crucial roles in microbial defense, representing evolutionarily ancient innate immune effectors. β-defensins are among the most extensively studied and widely distributed AMPs, functioning both as direct antimicrobial agents and immune modulators. Recent studies have identified multiple β-defensins expressed in porcine intestinal epithelium. The IPEC-J2 cell line, derived from newborn piglet jejunal epithelium, retains intestinal cell characteristics including β-defensin expression, making it suitable for studying nutritional and microbial stress effects on AMP expression.

Endogenous AMP expression and activity are regulated by various factors including bacteria, LPS, nutrient availability, and functional nutrients. While some studies in insects reported that starvation increases defensin expression, others found no effect of starvation but observed AMP induction by *E. coli* in tsetse flies. Our results differ from these findings: amino acid starvation significantly reduced defensin expression in IPEC-J2 cells, whereas *E. coli* had no significant effect. The *E. coli* concentration used in our study (10^3 CFU/mL) was substantially lower than that in the tsetse fly study (10 mL of culture at OD600 = 0.5), which may explain the discrepancy. Research on starvation and bacterial effects on mammalian AMPs remains limited, and variations across species, experimental systems (in vivo vs. in vitro), and defensin types contribute to these divergent results, warranting further investigation.

Recent studies have demonstrated that amino acids can promote β-defensin expression in various epithelial cells. Isoleucine at 3.12–12.50 g/mL significantly induced β-defensin expression in bovine kidney epithelial cells, though this effect

diminished at concentrations $ $25 g/mL. Similarly, 100–250 g/mL isoleucine promoted human β-defensin 1 expression in human colon cancer cells (HCT-116), and 5–50 g/mL isoleucine increased human β-defensin 2 expression in Caco-2 cells. Our finding that 1.0 mmol/L isoleucine (approximately 131 g/mL) significantly enhanced β-defensin expression aligns with these reports. The lack of effect from nitrogen-equivalent alanine confirms that isoleucine' s action is specific rather than simply providing nitrogen.

Both nutritional stress through amino acid starvation and amino acid supplementation represent nutritional modulation strategies. Multiple signaling pathways regulate AMP expression in animals, including NF- B, ERK, Sirt, and FoxO. The NF- B pathway primarily mediates bacterial, LPS, and infection effects on AMP expression; however, we did not examine this pathway since *E. coli* did not significantly affect defensin expression. Amino acid-related signaling pathways include MAPK, Sirt, mTOR, and FoxO, suggesting that amino acids may regulate AMP expression through Sirt and FoxO pathways.

Sirt1 is an NAD^+-dependent deacetylase, the mammalian homolog of yeast Sir2, traditionally studied for its role in lifespan regulation. Emerging evidence identifies Sirt1 as a crucial nutrient-sensitive growth regulator. Branched-chain amino acids, including isoleucine, can affect Sirt1 expression in rats. The FoxO family, a subgroup of forkhead proteins conserved from worms to humans, comprises four mammalian genes (FoxO1, FoxO3, FoxO4, and FoxO6) that regulate cell proliferation, immune responses, and longevity. Stress and nutrients can modulate Sirt1 through FoxO-dependent pathways, while Sirt1 directly influences defensin expression. Our results show that nutritional stress reduced both defensin and signaling protein expression, whereas isoleucine markedly enhanced defensin, Sirt1, and FoxO expression, with particularly strong effects on Sirt1. Alanine increased pEP2c, Sirt1, and FoxO4 expression to a lesser extent. These findings support previous research and suggest that nutritional stress and amino acid regulation of defensin expression involve Sirt1 and FoxO signaling pathways, though the specific regulatory mechanisms for different amino acids require further investigation.

In conclusion, nutritional stress decreases β-defensin expression in porcine intestinal epithelial cells, while amino acid supplementation promotes β-defensin expression, with isoleucine showing the most pronounced effect. The regulatory actions of nutritional stress and amino acids on defensins appear to be mediated through Sirt1 and FoxO signaling pathway proteins.

References

[1] ZHU Y G, JOHNSON T A, SU J Q, et al. Diverse and abundant antibiotic resistance genes in Chinese swine farms[J]. Proceedings of the National Academy of Sciences of the United States of America, 2013, 110(9): 3435–3440.

[2] LOOFT T, JOHNSON T A, ALLEN H K, et al. In-feed antibiotic effects on the swine intestinal microbiome[J]. Proceedings of the National Academy of Sciences of the United States of America, 2012, 109(5): 1691-1696.

[3] VELDHUIZEN E J A, RIJNDERS M, CLAASSEN E A, et al. Porcine β-defensin 2 displays broad antimicrobial activity against pathogenic intestinal bacteria[J]. Molecular Immunology, 2008, 45(2): 386-394.

[4] GALLO R L, HOOPER L V. Epithelial antimicrobial defence of the skin and intestine[J]. Nature Reviews Immunology, 2012, 12(7): 503-516.

[5] REN M, HUO Yingfeng, YANG Fengjuan, et al. Changes in intestinal morphology and related immune protein gene expression in piglets before and after weaning[J]. Chinese Journal of Animal Nutrition, 2014, 26(3): 614-619.

[6] DI NARDO A, BRAFF M H, TAYLOR K R, et al. Cathelicidin antimicrobial peptides block dendritic cell TLR4 activation and allergic contact sensitization[J]. Journal of Immunology, 2007, 178(3): 1829-1834.

[7] ZENG X F, SUNKARA L T, JIANG W Y, et al. Induction of porcine host defense peptide gene expression by short-chain fatty acids and their analogs[J]. PLoS One, 2013, 8(8): E72922.

[8] REN M, ZHANG S H, ZENG X F, et al. Branched-chain amino acids are beneficial to maintain growth performance and intestinal immune-related function in weaned piglets fed protein restricted diet[J]. Asian-Australasian Journal of Animal Sciences, 2015, 28(12): 1742-1750.

[9] ZASLOFF M. Antimicrobial peptides in multicellular organisms[J]. Nature, 2002, 415(6870): 389-395.

[10] SANTAOLALLA R, FUKATA M, ABREU M T. Innate immunity in the small intestine[J]. Current Opinion in Gastroenterology, 2011, 27(2): 125-131.

[11] MAO X B, QI S, YU B, et al. Dietary L-arginine supplementation enhances porcine β-defensins expression in tissues of weaned pigs[J]. Livestock Science, 2012, 148(1/2): 103-108.

[12] MAO X B, QI S, YU B, et al. Zn^{2+} and L-isoleucine induce the expressions of porcine β-defensins in IPEC-J2 cells[J]. Molecular Biology Reports, 2013, 40(2): 1547-1552.

[13] MUTURI E J, KIM C H, ALTO B W, et al. Larval environmental stress alters Aedes aegypti competence for Sindbis virus[J]. Tropical Medicine & International Health, 2011, 16(8): 955-964.

[14] BECKER T, LOCH G, BEYER M, et al. FOXO-dependent regulation of innate immune homeostasis[J]. Nature, 2010, 463(7279): 369-373.

[15] AKODA K, VAN DEN BOSSCHE P, MARCOTTY T, et al. Nutritional stress affects the tsetse fly' s immune gene expression[J]. Medical and Veterinary Entomology, 2009, 23(3): 195-201.

[16] FEHLBAUM P, RAO M, ZASLOFF M, et al. An essential amino acid induces epithelial β-defensin expression[J]. Proceedings of the National Academy of Sciences of the United States of America, 2000, 97(23): 12723–12728.

[17] SHERMAN H, CHAPNIK N, FROY O. Albumin and amino acids upregulate the expression of human beta-defensin 1[J]. Molecular Immunology, 2006, 43(10): 1617–1623.

[18] KONNO Y, ASHIDA T, INABA Y, et al. Isoleucine, an essential amino acid, induces the expression of human β-defensin 2 through the activation of the G-protein coupled receptor-ERK pathway in intestinal epithelia[J]. Food and Nutrition Sciences, 2012, 3(4): 548–555.

[19] WEHKAMP J, HARDER J, WEHKAMP K, et al. NF- B- and AP-1-mediated induction of human beta defensin-2 in intestinal epithelial cells by Escherichia coli Nissle 1917: a novel effect of a probiotic bacterium[J]. Infection and Immunity, 2004, 72(10): 5750–5758.

[20] YONEDA J, ANDOU A, TAKEHANA K. Regulatory roles of amino acids in immune response[J]. Current Rheumatology Reviews, 2009, 5(4): 252–258.

[21] DE SIMONE R, VISSICCHIO F, MINGARELLI C, et al. Branched-chain amino acids influence immune properties of microglial cells and their responsiveness to pro-inflammatory signals[J]. Biochimica et Biophysica Acta: Molecular Basis of Disease, 2013, 1832(5): 650–659.

[22] TANNO M, SAKAMOTO J, MIURA T, et al. Nucleocytoplasmic shuttling of the NAD^+-dependent histone deacetylase SIRT1[J]. Journal of Biological Chemistry, 2006, 282(9): 6823–6832.

[23] NARALA S R, ALLSOPP R C, WELLS T B, et al. SIRT1 acts as a nutrient-sensitive growth suppressor and is associated with increased AMPK and telomerase activity[J]. Molecular Biology of the Cell, 2008, 19(3): 1210–1219.

[24] D' ANTONA G, RAGNI M, CARDILE A, et al. Branched-chain amino acid supplementation promotes survival and supports cardiac and skeletal muscle mitochondrial biogenesis in middle-aged mice[J]. Cell Metabolism, 2010, 12(4): 362–374.

[25] JONSSON H, PENG S L. Forkhead transcription factors in immunology[J]. Cellular and Molecular Life Sciences, 2005, 62(4): 397–409.

[26] NEMOTO S, FERGUSSON M M, FINKEL T. Nutrient availability regulates SIRT1 through a forkhead-dependent pathway[J]. Science, 2004, 306(5704): 2105–2108.

[27] LEE S I, MIN K S, BAE W J, et al. Role of SIRT1 in heat stress- and lipopolysaccharide-induced immune and defense gene expression in human dental pulp cells[J]. Journal of Endodontics, 2011, 37(11): 1525–1530.

Corresponding author, Professor, E-mail: qiaoshy@mafic.ac.cn