https://doi.org/10.17221/213/2022-CJAS

Gly-Leu instead of Gly promoted the proliferation and protein synthesis of chicken intestinal epithelial cells

*Jing Liang, Baolong Du, Minyan Wan, Yapeng Zhao, Defu Tang**

Department of Animal Nutrition and Feed Science, College of Animal Science and Technology, Gansu Agricultural University, Lanzhou, P.R. China

**Corresponding author: defutang@126.com*

Citation: Liang J., Du B.L., Wan M.Y., Zhao Y.P., Tang D.F. (2023): Gly-Leu instead of Gly promoted the proliferation and protein synthesis of chicken intestinal epithelial cells. Czech J. Anim. Sci., 68: 391–398.

Abstract: Amino acids have positive regulatory effects on the function of intestinal epithelial cells (IEC), but in the field of animal nutrition, research on the regulatory effects of amino acids on IEC is still in the initial stages. This study aims to explore the effects of Gly, Gly-Gly, and Gly-Leu on IEC proliferation and their possible mechanisms. Chicken small intestinal epithelial cells were separated using the tissue block method, and other miscellaneous cells were removed for digestion and passage culture. The IEC were cultured in the medium containing 20 nmol/l Gly, Gly-Gly and Gly-Leu for 24 h, and the expression of enterokinase and cytokeratin in cells, the growth curve and activity of IEC, cell cycle, differentially expressed genes, mRNA expression, and protein expression levels of p-mTOR and p-S6K1 in IEC were detected. Enterokinase and cytokeratin were expressed specifically in IEC. The results of growth curve and MTT revealed that the cell viability of IEC was significantly increased after treatment with Gly, Gly-Gly and Gly-Leu. The cell cycle results showed that compared with the control group, Gly, Gly-Gly and Gly-Leu intervention could increase the proportion of IEC in G1 phase, and the proportion in S phase of IEC was decreased. Transcriptome sequencing showed that compared with the control group, there were 54, 28 and 30 differential genes in Gly group, Gly-Gly group and Gly-Leu group, respectively. These genes were mainly enriched in nitric oxide synthesis and protein kinase B signalling, PI3K signal and cellular amino acid biosynthesis and transport signal pathways. RT-PCR results showed that the mRNA expression levels of PCYT2, SPP1, EMC6, GRIA2 and PKD2 were consistent with the sequencing results. Western blot results showed that compared with the control group, the protein expression of p-mTOR and p-S6K1 in Gly group, Gly-Gly group and Gly-Leu group was significantly increased. Gly-Leu can promote the protein synthesis in IEC by activating protein synthesis of mTOR signalling pathway in chicken IEC.

Keywords: small peptide; animal nutrition; protein expression; signal pathway

Broilers are the most commonly consumed meat in people's daily diet, and amino acids have a regulatory effect on the nutrition of broilers, which could improve the nutritional composition of broiler intestines to some extent, which is of great significance for studying the intestinal nutrition of broilers. Small peptides are compounds connecting two or more amino acids linked by peptide bonds, and are among the nutrients that have received renewed attention in recent years. It has been proved that it could improve the immune

Supported by the National Natural Science Foundation of China under Grants No. 31660664 and No. 31702123, Discipline Team Project of Gansu Agricultural University and Fuxi Young Talent Cultivation Project of Gansu Agricultural University under Grant No. GAUFX-02Y07, and Discipline Team Project of Gansu Agricultural University No. GAU-XKTD-2022-24.

https://doi.org/10.17221/213/2022-CJAS

system of animals, promote the reproduction of beneficial bacteria in the body, and promote protein synthesis and nitrogen balance (Souba 1992). Proteins are degraded by trypsin and chymotrypsin in the intestine into free amino acids and small peptides, which are completely hydrolysed into free amino acids under the action of peptidase and enter the circulatory system as free amino acids (Terada and Inui 2012). Dietary supplementation of methionine and methionine dipeptide alleviated the effects of heat stress on broiler metabolism by upregulating the activation of genes related to the glutathione antioxidant system (Santana et al. 2021). Hu et al. (2017) found that supplementation of leucine could induce the reduction of ROS and the switch of energy metabolism in IPEC-J2 cells. Phenylalanine-proline dipeptide could significantly downregulate the expression of ABCA1 in the rat jejunum to improve cholesterol metabolism (Banno et al. 2019). It could be seen that the absorption of small peptides in intestinal cells plays a key role in the whole process of protein metabolism in animals.

Leucine is not only a substrate for protein synthesis, but also a signal molecule involved in protein metabolism, mediating various biological processes such as protein synthesis, degradation, fatty acid oxidation and insulin secretion (Yang et al. 2012; Wu 2013). Leucine can promote protein synthesis in muscle tissue and IEC through the mTOR signal pathway (Deng et al. 2014; Nakai et al. 2018). Glycine is a conditionally essential amino acid for mammals, which is particularly important for the body nutrition. In addition, glycine can also protect organs such as liver, kidney, lung, skeletal muscle and intestine from harmful substances (Lee et al. 2001; Tsune et al. 2003; Wang et al. 2014a). However, the specific effects and mechanisms of glycine on renewal and protein synthesis of IEC are still unknown. At present, studies on small peptides at home and abroad mainly focus on traditional nutrition and small peptide intestinal transporters, but there have been few reports on small peptide metabolism regulatory pathways at cellular and molecular levels, which limits the development and understanding of "small peptide nutrition theory" to a certain extent. It is of important scientific significance to study the effects of small peptides on broiler IEC and their regulatory pathways, and to enrich and perfect the nutritional theory system of small peptides. Therefore, we used IEC of broilers to comprehensively compare the renewal and protein conversion of IEC by Gly, Gly-Gly and Gly-Leu through MTT, flow cytometry, RT-PCR, western blot and transcriptome sequencing.

MATERIAL AND METHODS

Isolation and culture of chicken small intestinal epithelial cells

The small intestine of broiler embryos aged 18 to 19 was taken out in a super-clean workbench. The small intestine was put into a centrifuge tube containing penicillin and streptomycin. The small intestine was cleaned with PBS (P1022; Solarbio, Beijing, China), and the adipocytes and mesentery were removed and cut into fragments. The tissue blocks were suspended in serum-free DMEM/F12 (31330095; Gibco, Billings, MT, USA) medium and centrifuged at 1 000 RPM for 4 minutes. Discard the supernatant, clean the intestinal tissue to clarify the supernatant, add DMEM/F12 to complete the medium suspension tissue block containing 10% FBS (23022-8615; Sijiqing, Pricella, Wuhan, China), transfer the intestinal tissue to a 6-well plate, and culture in a 5% CO_2 incubator. About 8 h later, intestinal epithelial cells appear around the tissue. The intestinal epithelial cell colonies were observed under an inverted microscope, and the rest of the miscellaneous cells were removed. The cells were separated and digested with trypsin, resuspended into a single cell suspension, adjusted to a cell density of 500 cells/ml, 100 µl were inoculated into a 96-well plate, and cultured for a week.

Identification of IEC

The IEC were inoculated in a 6-well plate, and the total RNA was extracted with Trizol (15596026; Invitrogen, Waltham, MA, USA) when the cell density reached 80%. The ratio of A260/280 was measured by an ultra-micro spectrophotometer in the range of 1.8—2.0. Reverse transcription was performed with a reverse transcription kit (RR047A; Takara Bio, Inc., Kusacu, Japan), and the obtained cDNA was used as a template to amplify the target gene. There are three replicates for each reaction.

MTT assay

MTT was used to detect cell viability. The cell density was adjusted to 1.5×10^5 cells/ml, and IEC were re-inoculated into a 96-well plate. IEC were starved in DMEM-F12 medium for 24 h, then the cells were cultured in the same medium containing 20 nmol/l

https://doi.org/10.17221/213/2022-CJAS

Gly, Gly-Gly and Gly-Leu for 24 h, and the untreated cells were used as blank control group. The supernatant in the well was discarded, and MTT solution (M8180; Solabio, Beijing, China) was added to each well and incubated for 4 h. 150 µl DMSO (D8371; Solabio, Beijing, China) was added to dissolve with vibration. The OD value was detected at 490 nm with the microplate reader.

Flow cytometry

Cells at logarithmic growth stage were taken and treated with 20 nmol/l Gly, Gly-Gly and Gly-Leu, and cells without any treatment were used as blank control group. The cells were collected after being treated for 24 h, and were resuspended with PBS to adjust the cell density of 1×10^6 cells/ml. After centrifugation, the supernatant was discarded, and the precipitation was fixed with 70% precooled ethanol for 2 h. After the cells were washed with PBS, RNase A (Sigma, R6513) solution was added to resuscitate the cells, and the cells were incubated in a water bath at 37 °C for 30 minutes. PI was added into the cells and mixed. The cells were incubated at 4 °C for 30 min under dark conditions and detected by flow cytometry.

Transcriptome sequencing

After IEC were treated with 20 nmol/l Gly, Gly-Gly and Gly-Leu, the cells were collected and sent to Hangzhou Lianchuan Bioinformatics Technology Co., Ltd. for mRNA transcription profile analysis. GO functional enrichment and KEGG pathway enrichment were performed to screen differentially expressed genes related to protein synthesis, and their expression levels were detected by RT-PCR.

RT-PCR

The IEC from each group were collected and the mRNA expression of mTOR, 4EBP1 and S6K1 in IEC was detected by RT-PCR.

Statistical analysis

IBM SPSS v21.0 (IBM Corp., Armonk, NY, USA) software was used for statistical analysis of the experimental data, and the measurement data was expressed as mean ± SD. LSD *t*-test was used to make pairwise comparisons between groups, and single factor analysis was used to compare multiple groups. $P < 0.05$ or $P < 0.01$ indicated that the difference was statistically significant.

RESULTS

Isolation and culture of IEC

The IEC that have been successfully isolated exhibit different morphologies. The cells are closely connected with each other and grow in the shape of a single layer of paving stones and tadpoles (Figure 1A). Enterokinase and cytokeratin are the signature proteins of IEC. We examined their mRNA expression levels and found that enterokinase and cytokeratin were specifically expressed in IEC (Figure 1B).

Effect of small peptides on the growth of IEC

The growth curve of IEC was measured, and it was found that the IEC in the control group grew slowly on the first day, grew faster from the 2nd to 4th day, and entered the logarithmic growth phase. From day 4 to 6, the growth slowed down and reached the plateau phase. The growth curve of IEC treated with Gly, Gly-Gly and Gly-Leu showed the same trend as that of the control group (Figure 2A). In addition, the viability of IEC was also detected. Compared with the control group, the viability of IEC in Gly group, Gly-Gly group and Gly-Leu group was significantly increased ($P < 0.05$). Compared with Gly group and Gly-Gly group, the viability of IEC in Gly-Leu group was significantly increased ($P < 0.05$, Figure 2B). The cell cycle analysis showed that compared with the control group, the proportion of cells in the S phase in Gly group, Gly-Gly group and Gly-Leu group was decreased, while and the proportion of G1 phase cells was increased ($P < 0.05$, Figure 2C and Figure 2D).

Differential gene expression and functional enrichment analysis

We performed the mRNA transcription profiling of IEC and we found that compared with control

https://doi.org/10.17221/213/2022-CJAS

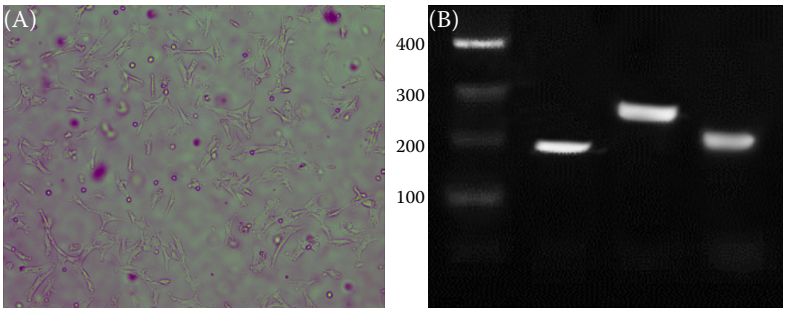

Figure 1. IEC cell morphology and specific gene expression analysis
(A) IEC cell morphology, 200×; (B) agarose gel electrophoresis. From left to right in the figure are DNA marker, enterokinase, and cytokeratin in sequence

group, there were 54, 28 and 29, and there were 13 differentially expressed genes in the Gly-Leu group (Figure 3A).

The GO function enrichment analysis of differentially expressed genes revealed that differentially expressed genes were enriched in biological processes, cell components and molecular functions, and they were mainly enriched in the biosynthesis of serine, positive regulation of nitric oxide biosynthesis, positive regulation of protein kinase B signal, positive regulation of PI3K signal, phosphatidylinositol transport activity, cellular

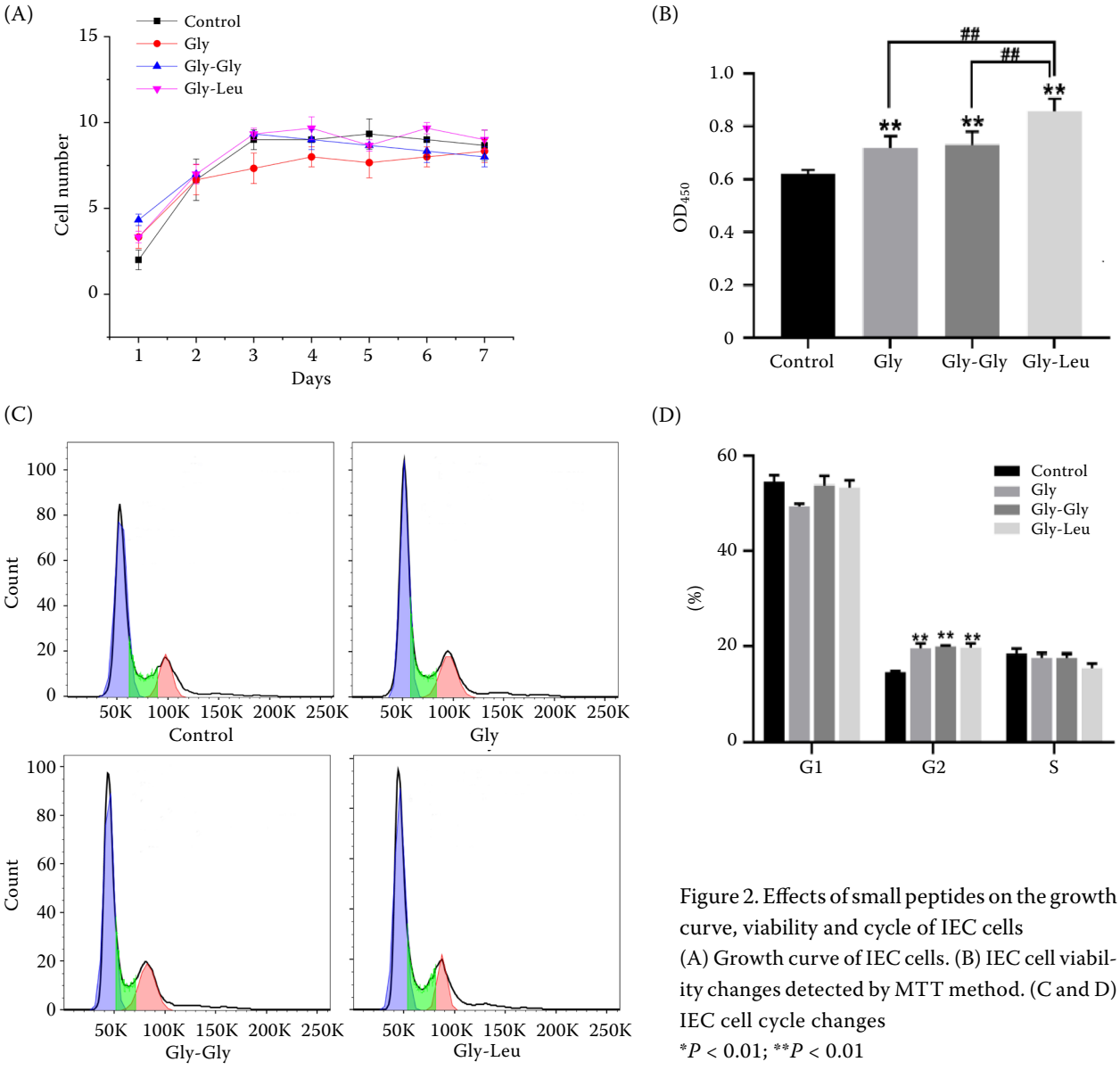

Figure 2. Effects of small peptides on the growth curve, viability and cycle of IEC cells
(A) Growth curve of IEC cells. (B) IEC cell viability changes detected by MTT method. (C and D) IEC cell cycle changes
*P < 0.01; **P < 0.01

https://doi.org/10.17221/213/2022-CJAS

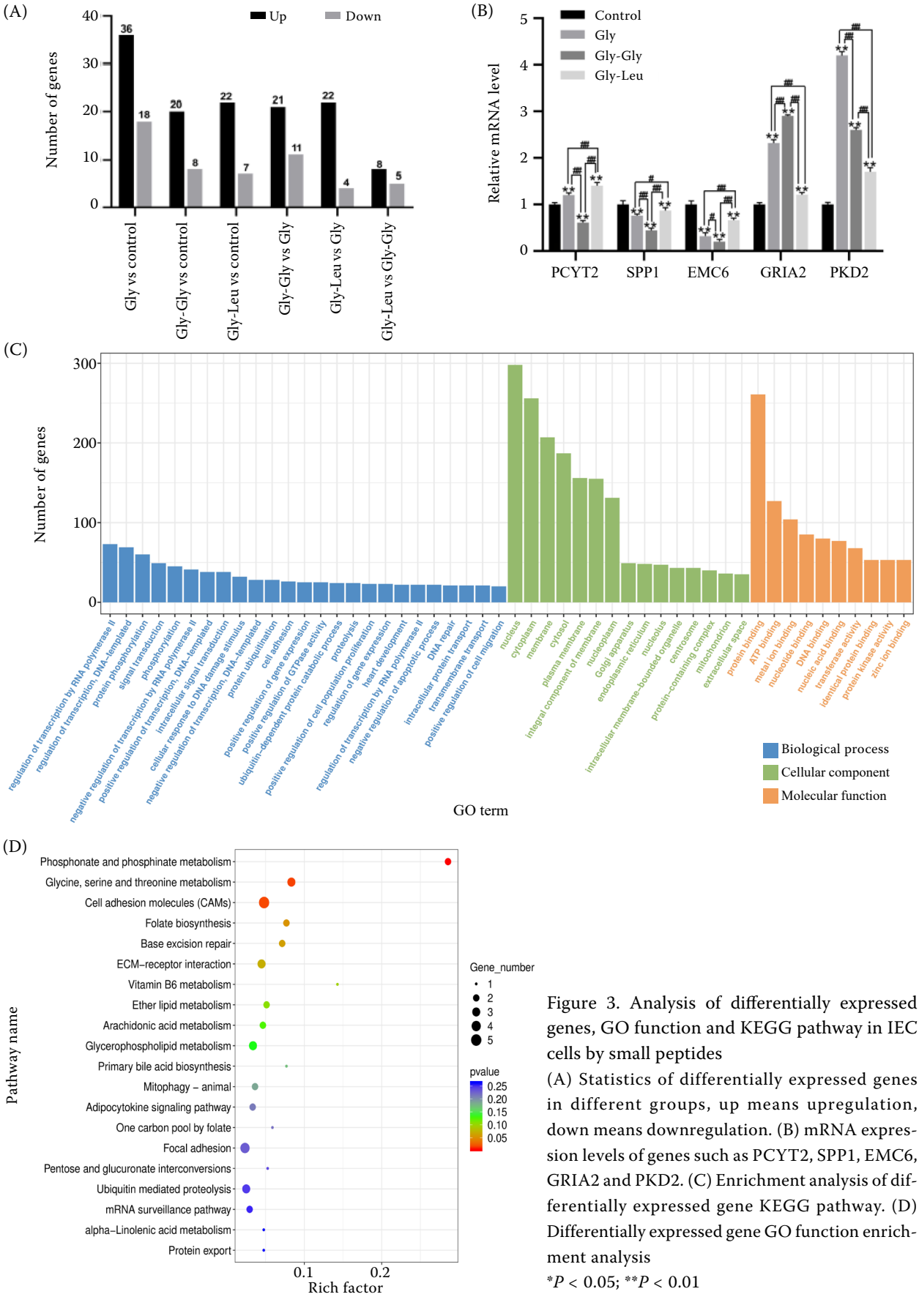

Figure 3. Analysis of differentially expressed genes, GO function and KEGG pathway in IEC cells by small peptides

(A) Statistics of differentially expressed genes in different groups, up means upregulation, down means downregulation. (B) mRNA expression levels of genes such as PCYT2, SPP1, EMC6, GRIA2 and PKD2. (C) Enrichment analysis of differentially expressed gene KEGG pathway. (D) Differentially expressed gene GO function enrichment analysis

*$P < 0.05$; **$P < 0.01$

https://doi.org/10.17221/213/2022-CJAS

amino acid biosynthesis, amino acid transport, signal receptor pathway regulation and cell cycle regulation (Figure 3C). KEGG pathway analysis shows that these differentially expressed genes were mainly enriched in glycine, serine, threonine, alanine, tyrosine and other amino acid metabolism, adhesion plaques, protein synthesis in the endoplasmic reticulum, cell cycle, ErbB signal, VEGF, MAPK and mTOR signalling pathways (Figure 3D).

In order to verify the differential gene expression screened by transcriptome sequencing analysis, we performed RT-PCR verification on the expression of differentially expressed genes such as PCYT2, SPP1, EMC6, GRIA2, and PKD2. As shown in Figure 3B, the expression levels of these differentially expressed genes are consistent with the transcriptome sequencing results.

The influence of small peptides on IEC mTOR signal

The RT-PCR method was used to detect the mRNA expression levels of mTOR, 4EBP1 and S6K1. The results showed that compared with the control group, the mRNA expressions of mTOR, 4EBP1 and S6K1 in Gly group, Gly-Gly group and Gly-Leu group were significantly increased ($P < 0.05$). Compared with Gly group, the mRNA expressions of mTOR, 4EBP1 and S6K1 in Gly-Gly group and Gly-Leu group were significantly increased ($P < 0.05$, Figure 4).

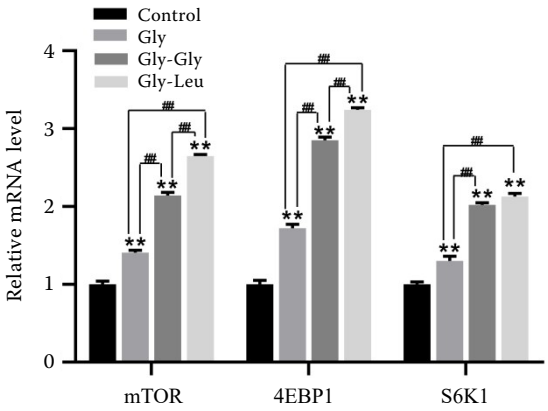

Figure 4. The effect of small peptides on the mRNA expression of mTOR, 4EBP1 and S6K1 in IEC cells
*$P < 0.05$; **$P < 0.01$

DISCUSSION

Many studies were based on intestinal epithelial cells for nutritional and physiological research in the intestine (Kaiser et al. 2017; Tokutake et al. 2021). Therefore, the breakdown and metabolism of amino acids by intestinal epithelial cells can provide important references for studying intestinal amino acid metabolism. Research has confirmed that arginine, leucine, and alanine can be used to synthesize proteins by intestinal epithelial cells (Yin et al. 2010; Bertrand et al. 2013). This study synthesized Gly-Gly and Gly-Leu using glycine and leucine as raw materials to intervene in IEC, and explored the effects of Gly-Gly and Gly-Leu on IEC renewal and protein synthesis.

Studies have revealed that Gly can promote the proliferation and protein synthesis of IEC, similarly to Wang's research findings, which indicate that Gly plays an important role in protein metabolism. Diets supplemented with different contents of Gly can significantly increase the daily gain of piglets, and decrease the contents of nitrogen and urea in serum, thus improving the utilization of nitrogen (Wang et al. 2014b). After the intestinal epithelial cells were treated with different concentrations of Gly, it was found that the proliferation of intestinal epithelial cells depended on the concentration of Gly, and could reduce the oxidative stress level of cells, activate mTOR signal, and promote protein synthesis of intestinal epithelial cells (Wang et al. 2014a). In addition, the results of this study also demonstrated that Gly-Gly and Gly-Leu, similarly like Gly, could be used as nutritional signal regulators to promote the synthesis of IEC proteins. The effect of small peptides compared with Gly indicated that small peptides had a stronger role in promoting effect on IEC proliferation and protein synthesis.

Animal growth signal and nutrient sensing are integrated through a series of signalling pathways, among which mTOR signal is the most important cell signal. mTOR is involved in corresponding responses according to the nutritional conditions of the cell environment, and participates in regulating the activities of protein kinases and protein phosphatases, thereby controlling the expression of genes related to protein and gene transcription (Cao et al. 2019). When nutrients or other growth stimuli appear, cells actively upregulate the synthesis of macromolecular substances to promote cell growth. Conversely,

https://doi.org/10.17221/213/2022-CJAS

cells can inhibit the synthesis of macromolecules and increase the material turnover in response to nutrient restriction or other stresses (Wang and Proud 2006). It was found that amino acids could act directly on mTOR as signal molecules to activate mTOR. Activated mTOR further promotes the activation of downstream factors 4EBP1 and S6K1. Activation of S6K1 can promote the phosphorylation of 4EBP1 and ribosomal protein S6K1, and further promote the translation process of certain specific mRNAs. These specific proteins often participate in the translation mechanism of mRNA (Cao et al. 2019). Amino acids and small peptides have been found to activate S6K1 and 4EBP1 (Drummond et al. 2008; Yang 2017). In the previous study, we also found that dietary leucine significantly upregulated the expression levels of mTOR, S6K1 and 4EBP1 mRNA and phosphorylation levels of mTOR and S6K1 in breast muscle, thereby activating the mTOR signalling pathway. In this study, we found that Gly, Gly-Gly, and Gly-Leu could promote the expression of mTOR and its downstream signalling molecules S6K1 and 4EBP1, suggesting that they could stimulate protein synthesis in IEC by activating the mTOR signalling pathway.

At the same time, small peptides can also play a role as signal molecules. Researchers have discovered that small peptides such as L-Tyr-Arg, β-Ala-His, Cys-Gly, and Gly-Gln, etc. can be used as signal molecules to participate in the signal transduction of the central nervous system. Therefore, it is highly likely that small peptides are involved in the regulation of protein synthesis in IEC as signalling molecules in the small intestine. Differential gene expression in IEC after dipeptide intervention was determined using transcriptome sequencing. We found that compared with Gly group, there were 21 and 17 upregulated differential genes in IEC in Gly-Gly group and Gly-Leu group, and 11 and 10 downregulated differential genes in IEC in Gly-Gly group and Gly-Leu group, respectively. Among them, ENSGALG00000047524, ENSGALG00000054180, ENSGALG00000047563, ENSGALG00000007080 and RXFP1 were significantly upregulated in these two groups. Compared with Gly-Gly group, there were eight upregulated differential genes and five downregulated genes in Gly-Leu group. In addition, the expression levels of five differentially expressed genes in IEC were detected by RT-PCR, and the results were consistent with the sequencing results. Further functional analysis of differential genes showed that these differential genes were mainly enriched in biological processes, but also affected the expression of related genes in molecular functions and cellular components. From the analysis of the pathways involved, differential genes were mainly enriched in phosphonate and phosphonate metabolism, glycerol phospholipid metabolism, apelin signalling pathway and focal adhesion, toll-like receptor signalling pathway, cytokine-cytokine receptor interaction, and phosphatidylinositol signalling signals of system and glycerolipid metabolism. When Gly, Gly-Gly and Gly-Leu act on IEC, they may promote protein synthesis by upregulating the pathway factors in the above related signals. In these pathways, which dominates, and how to coordinate the protein synthesis process in IEC, this remains to be further studied. Further research is needed to determine which of these pathways are dominant and how they coordinate the protein synthesis process in IEC.

In summary, our research shows that Gly, Gly-Gly and Gly-Leu can promote IEC proliferation and protein synthesis by upregulating the genes of the three main effector elements mTOR, 4EBP1 and S6K1 in the mTOR signalling pathway. In addition, phosphonate and phosphonate metabolism, glycerol phospholipid metabolism, apelin signalling pathway, focal adhesion, toll-like receptor signalling pathway, cytokine-cytokine receptor interaction, phosphatidylinositol signalling system, and glycerolipid metabolism also participate in this process.

Conflict of interest

The authors declare no conflict of interest.

REFERENCES

Banno A, Wang JL, Okada K, Mori R, Mijiti M, Nagaoka S. Identification of a novel cholesterol-lowering dipeptide, phenylalanine-proline (FP), and its down-regulation of intestinal ABCA1 in hypercholesterolemic rats and Caco-2 cells. Sci Rep. 2019 Dec 19;9: 12 p.

Bertrand J, Goichon AD, Chelotte P, Coeffier M. Regulation of intestinal protein metabolism by amino acids. Amino Acids. 2013 Sep 11;45(3):443-50.

Cao YC, Liu SM, Liu K, Abbasi IHR, Cai CJ, Yao JH. Molecular mechanisms relating to amino acid regulation of protein synthesis. Nutr Res Rev. 2019 Dec;32(2):183-91.

https://doi.org/10.17221/213/2022-CJAS

Deng HL, Zheng AJ, Liu GH, Chang WH, Zhang S, Cai HY. Activation of mammalian target of rapamycin signaling in skeletal muscle of neonatal chicks: Effects of dietary leucine and age. Poult. 2014 Jan;93(1):114-21.

Drummond MJ, Bell JA, Fujita S, Dreyer HC, Glynn EL, Volpi E, Rasmussen BB. Amino acids are necessary for the insulin-induced activation of mTOR/S6K1 signaling and protein synthesis in healthy and insulin resistant human skeletal muscle. Clin Nutr. 2008 Jun;27(3):447-56.

Hu J, Nie YF, Chen SF, Xie CL, Fan QW, Wang ZC, Long BS, Yan GK, Zhong Q, Yan XH. Leucine reduces reactive oxygen species levels via an energy metabolism switch by activation of the mTOR-HIF-1α pathway in porcine intestinal epithelial cells. Int J Biochem Cell Biol. 2017 Aug;89:42-56.

Kaiser A, Willer T, Steinberg P, Rautenschlein S. Establishment of an in vitro intestinal epithelial cell culture model of avian origin. Avian Dis. 2017 Jun;61(2):229-36.

Lee MA, McCauley RD, Kong SE, Hall JC. Pretreatment with glycine reduces the severity of warm intestinal ischemic-reperfusion injury in the rat. Ann Plast Surg. 2001 Mar;46(3):320-6.

Nakai N, Kawano F, Murakami T, Nakata K, Higashida K. Leucine supplementation after mechanical stimulation activates protein synthesis via L-type amino acid transporter 1 in vitro. J Cell Biochem. 2018 Feb;119(2):2094-101.

Santana TP, Gasparino E, de Sousa FC, Khatlab AS, Zancanela V, Brito CO, Barbosa LT, Fernandes RPM, Del Vesco AP. Effects of free and dipeptide forms of methionine supplementation on oxidative metabolism of broilers under high temperature. Animal. 2021 Mar;15(3): 7 p.

Souba WW. Gultamine: Physiology, biochemistry and nutrition in critical illness. Austin, Boca Raton: CRC Press; 1992.

Terada T, Inui K. Recent advances in structural biology of peptide transporters. In: Bevensee M, editor. Co-transport systems. San Diego: Elsevier; 2012. p. 257-74. 70 vol.

Tokutake Y, Taciak M, Sato K, Toyomizu M, Kikusato M. Effect of dipeptide on intestinal peptide transporter 1 gene expression: An evaluation using primary cultured chicken intestinal epithelial cells. Anim Sci J. 2021 Dec; 92(1): 6 p.

Tsune I, Ikejima K, Hirose M, Yoshikawa M, Enomoto N, Takei Y, Sato N. Dietary glycine prevents chemical-induced experimental colitis in the rat. Gastroenterology. 2003 Sep;125(3):775-85.

Wang XM, Proud CG. The mTOR pathway in the control of protein synthesis. Physiology. 2006 Oct;21(5):362-9.

Wang WW, Dai ZL, Wu ZL, Lin G, Jia S, Hu SD, Dahanayaka S, Wu GY. Glycine is a nutritionally essential amino acid for maximal growth of milk-fed young pigs. Amino Acids. 2014a Aug;46(8):2037-45.

Wang WW, Wu ZL, Lin G, Hu SD, Wang B, Dai ZL, Wu GY. Glycine stimulates protein synthesis and inhibits oxidative stress in pig small intestinal epithelial cells. J Nutr. 2014b Oct;144(10):1540-8.

Wu GY. Functional amino acids in nutrition and health. Amino Acids. 2013 Sep;45(3):407-11.

Yang H. Structure of the FRB domain of mTOR bound to a substrate recruitment peptide of S6K1. Worldwide Protein Data Bank; 2017. Available from: https://doi.org/10.2210/pdb5WBH/pdb

Yang J, Dolinger M, Ritaccio G, Mazurkiewicz J, Conti D, Zhu XJ, Huang YF. Leucine stimulates insulin secretion via down-regulation of surface expression of adrenergic α2A receptor through the mTOR (Mammalian Target of Rapamycin) pathway IMPLICATION IN NEW-ONSET DIABETES IN RENAL TRANSPLANTATION. J Biol Chem. 2012 Jul 13;287(29):24795-806.

Yin YL, Yao K, Liu ZJ, Gong M, Ruan Z, Deng D, Tan B, Liu ZQ, Wu GY. Supplementing L-leucine to a low-protein diet increases tissue protein synthesis in weanling pigs. Amino Acids. 2010 Nov;39(5):1477-86.

Received: December 4, 2022
Accepted: July 19, 2023
Published online: September 26, 2023