Amino Acids (2017) 49:2023–2031
DOI 10.1007/s00726-017-2460-z

ORIGINAL ARTICLE

Alanyl-glutamine but not glycyl-glutamine improved the proliferation of enterocytes as glutamine substitution in vitro

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Received: 2 May 2017 / Accepted: 26 June 2017 / Published online: 31 August 2017

Abstract The synthetic dipeptides alanyl-glutamine (Ala-Gln) and glycyl-glutamine (Gly-Gln) are used as Gln substitution to provide energy source in the gastrointestinal tract due to their high solubility and stability. This study aimed to investigate the effects of Gln, Ala-Gln and Gly-Gln on mitochondrial respiration and protein turnover of enterocytes. Intestinal porcine epithelial cells (IPEC-J2) were cultured for 2 days in Dulbecco's modified Eagle's-F12 Ham medium (DMEM-F12) containing 2.5 mM Gln, Ala-Gln or Gly-Gln. Results from 5-ethynyl-2′-deoxyuridine incorporation and flow cytometry analysis indicated that there were no differences in proliferation between free Gln and Ala-Gln-treated cells, whereas Gly-Gln treatment inhibited the cell growth compared with Gln treatment. Significantly lower mRNA expressions of Sp1 and PepT1 were also observed in Gly-Gln-treated cells than that of Ala-Gln treatment. Ala-Gln treatment increased the basal respiration and ATP production, compared with free Gln and Gly-Gln treatments. There were no differences in protein turnover between free Gln and Ala-Gln-treated cells, but Gly-Gln treatment reduced protein synthesis and increased protein degradation. Ala-Gln treatment stimulated mTOR activation whereas Gly-Gln decreased mTOR phosphorylation and increased the UB protein expression compared with free Gln treatment. These results indicate that Ala-Gln has the very similar functional profile to free Gln in porcine enterocytes in vitro and can be substituted Gln as energy and protein sources in the gastrointestinal tract.

Keywords Glutamine · Dipeptide · Mitochondrial bioenergetics · Protein turnover · Enterocyte

Abbreviations

4EBP1	4E binding protein-1
AA	Amino acids
Ala-Gln	Alanyl-glutamine
ATP	Adenosine triphosphate
DMEM-F12	Dulbecco's modified Eagle's-F12 Ham medium
ECAR	Extracellular acidification rate
EdU	5′-Ethynyl-2′-deoxyuridine
FCCP	Carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
Gln	Glutamine
Gly-Gln	Glycyl-glutamine
IPEC	Intestinal porcine epithelial cell
mTOR	Mammalian target of rapamycin
OCR	Oxygen consumption rate
PepT1	Peptide transporter 1
S6K1	Ribosomal protein S6 kinase-1
Sp1	Specificity protein 1
UB	Ubiquitin

Handling Editors: C.-A.A. Hu, Y. Yin, Y. Hou, G. Wu, Y. Teng.

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Introduction

Glutamine (Gln) is considered to be a conditional essential amino acid (AA) as it can be synthesized by glutamine synthase from glutamate and ammonia under normal physiological conditions (Wise and Thompson 2010; Wu et al. 2011). Gln plays various physiological functions such as synthesis of protein and purines, maintenance of cellular energy, acid–base balance, immune function and inhibition of tumor growth (Aled 2004; Brosnan 2003; Newsholme 2001; Wise and Thompson 2010; Yuneva et al. 2007). However, Gln becomes essential under severe illness or injury when external supplementation of Gln is necessary (Wise and Thompson 2010; Wu et al. 2011).

Free Gln has low absorption rate in intestine, due to its low solubility and instability under acid environment; therefore, there is increasing interest in using Gln-containing peptides as an alternative for Gln supplementation in diet (Labow and Souba 2000). Steinhardt and Adibi (1986) found that except for arginine, all AA were absorbed significantly higher in the form of dipeptide than free AA. Gln dipeptide could be uptaken by intestinal mucosal cells through peptide transporter 1 (PepT1) and then quickly degraded into Gln and their respective free amino acid (Erickson and Kim 1990; Hu et al. 2008). However, it is likely that Gln-containing dipeptides might confer other biological functions different from free Gln. Hence, a proper evaluation of physiological functions of Gln versus its dipeptides is needed.

Alanyl-glutamine (Ala-Gln) and Glycyl-glutamine (Gly-Gln) are two well-known Gln-containing dipeptides (Cruzat and Tirapegui 2009; Wang et al. 2010). Supplementation of Ala-Gln was reported to improve protein metabolism and survival in rat bacterial peritonitis model (Naka et al. 1996), reduce infectious complications in surgical patients (Estivariz et al. 2008), reduce *Clostridium difficile* toxin-A induced intestinal epithelial cell damage (Santos et al. 2013), improve immune function and intestinal integrity in early weaned calves (Zhou et al. 2012), and provide protection against nelfinavir-induced epithelial impairment in IEC-6 cells as well as in mouse intestinal mucosa (Braga-Neto et al. 2012). Supplementation of Gly-Gln can also improve growth and intestinal integrity of weanling piglets by inhibiting inflammatory response in lipopolysaccharide-challenged piglets (Jiang et al. 2009), enhance mucosal structure and function after heterotopic small-bowel autotransplantation in the pig (Li et al. 2003) and increase the enzyme activity, cell proliferation and apoptosis of jejunal tissues from weaned piglets (Wang et al. 2011).

In this study, a comparison among free Gln, Ala-Gln and Gly-Gln was performed to determine their effects on intestinal epithelial cell renewal, mitochondrial respiration and protein turnover using porcine intestinal cell line IPEC-J2.

Materials and methods

Cell culture

IPEC-J2 (intestinal porcine epithelial cells J2) were cultured with DMEM-F12 medium (Hyclone, USA) containing 5% FBS (Gibico, USA), 1% antibiotic solution (P/S; Sigma, USA), 0.1% ITS (ScienCell, USA), and 5 μg/L mEGF (BD Biosciences, USA) at 37 °C in a 5% CO_2 incubator. After an overnight incubation, the cells were starved for 6 h in Gln-free DMEM-F12, and then incubated in same medium containing either 2.5 mM of Gln (as control), 2.5 mM of Ala-Gln, or 2.5 mM of Gly-Gln for 2 days. The cells cultured in Gln-free medium virtually did not proliferate, the treatment of 0 mM Gln was not included.

Cell growth analysis

Cell viability was determined using the Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Inc, Rockville, MD, USA) on a microplate reader using SpectraMax software (Molecular Devices, Inc. CA, USA) at 450 nm.

DNA synthesis was detected by the standard EdU incorporation method according to the protocol of Cell-Light™ EdU Kit (Rui Bo Biotechnology Limited Company, Guangzhou, China) and the previous study (Tan et al. 2015b). After labeling with EdU, cell fixation and permeabilization, EdU detection and DNA Staining, the cells were visualized under a Leica DMI 3000B fluorescent microscope (Leica, German). Images of the Apoll®567 Hoechst 33342 were captured. The percentage of EdU-positive cells was expressed as the ration of red nuclei cells to blue nuclei cells in at least five different microscopic fields randomly selected for counting at 100-fold magnification.

Cell cycle analysis was performed using iodide staining and detecting with flow cytometry (BD Biosciences, San Jose, CA, USA) at excitation and emission of 535 and 617 nm, respectively, as described by Tan et al. (2015b). The percentage of cells at G1, S and G2 phases of the cell cycle were analyzed with the CellQuest Pro software (Becton–Dickinson, CA, USA).

Mitochondrial function test

The XF-24 Extracellular Flux Analyzer and Cell Mito Stress Test Kit from Seahorse Biosciences (Billerica, MA, USA) were used to examine the mitochondrial respiration according to the descriptions in the previous studies (Tan et al. 2015a, b). After a 2-day period of culture, the growth medium were removed and cells were washed twice with 1000 μL of pre-warmed assay medium (XF Base Medium supplemented with 25 mM glucose, 2 mM glutamine and 1 mM sodium pyruvate; pH 7.4; Seahorse Bioscience Inc.,

Billerica, MA, USA). Cells were incubated in 37 °C incubator without CO_2 for 1 h to allow to pre-equilibrate with the above assay medium. Pre-warmed oligomycin, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP), rotenone & antimycin A at the final concentrations of 0.5, 1, and 1 μM were loaded into the injector ports A, B and C of sensor cartridge, respectively. The cartridge was calibrated by the XF24 analyzer (Seahorse Bioscience, Billerica, MA, USA), and the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were detected. This allowed for an estimation of the contribution of individual parameters for basal respiration, proton leak, maximal respiration, spare respiratory capacity, non-mitochondrial respiration and ATP production. The XF mito stress test report generator automatically calculate the XF cell mito stress test from wave data that have been exported to Excel. Total cellular protein was determined and used to normalize mitochondrial respiration rates.

Protein turnover determination

For protein synthesis determination, IPEC-J2 cells were cultivated in 6-well cell culture plates for 2 days, followed by washing with Gln-free medium, and then 2 mL of DMEM-F12 medium containing 1 mM [^{3}H] Phe (phenylalanine) (American Radiolabeled Chemicals, USA) and 2.5 mM of either Gln, Ala-Gln, or Gly-Gln. For protein degradation analysis, IPEC-J2 cells were cultivated for 1 day, followed by incubation with 2 mL of DMEM-F12 medium containing 1 mM [^{3}H] Phe and 2.5 mM of free Gln or Ala-Gln, or Gly-Gln for 24 h. After removal of medium, the cells were washed with 2 mL of DMEM-F12 medium containing 1 mM Phe and 2.5 mM of Gln or Ala-Gln, or Gly-Gln for three times. The next procedures were performed as described by Tan et al. (2010).

Real-time PCR

The protocol of total RNA extraction, quantification, cDNA synthesis and real-time PCR was according to the method of Li et al. (2015). Briefly, total RNA was extracted from cell samples using Trizol-reagent (Invitrogen) and the cDNA was reversed-transcribed from eluted RNA using the first strand cDNA synthesis kit (Fermentas). The real-time quantitative PCR for β-actin, Sp1 and PepT1 was carried out using the following primers to amplify genes: β-actin (F) 5′-GGACCTGACCGACTACCTCA-3′ (R) 5′-CACAGCTTCTCCTTGATGTCC-3′; Sp1 (F) 5′-ACAGCAGGTGGAGAAGGAGA-3′ (R) 5′-TCGGAGATGTGAGGTCTTGC-3′; PepT1 (F) 5′-AGGTTTAGGCATCGGAGTA-3′ (R) 5′-ATGGGAACCATGATAACGA-3′. The $2^{-\Delta\Delta CT}$ method was used to calculate the mRNA expression of the target genes relative to β-actin, and data are expressed relative to those in Gln treated cells.

Western blot

After a 2-day period of culture, cells were collected and the protein expressions of β-actin, mTOR, phosphorylated mTOR, 4EBP1, phosphorylated 4EBP1, S6K1, phosphorylated S6K1 and UB were determined as described previously (Tan et al. 2010). The following antibodies were used for protein quantification: β-actin (1:1000; Cell Signaling Technology, USA), mTOR (1:1000; Cell Signaling Technology, USA), phosphorylated mTOR (Ser2448) (1:1000; Cell Signaling Technology, USA), 4EBP1 (1:1000; Cell Signaling Technology, USA), phosphorylated 4EBP1 (Ser65) (1:1000; Cell Signaling Technology, USA), S6K1 (1:1000; Cell Signaling Technology, USA), phosphorylated S6K1 (Thr389) (1:1000; Cell Signaling Technology, USA), UB (1:1000; Abcam, USA). Data are expressed relative to the values of Gln-treated cells.

Statistical analysis

Statistical analyses were performed by one-way ANOVA using SPSS software 19.0 (SPSS Inc., Chicago, IL). The differences among treatments were evaluated using Tukey's test. Data are presented as the mean ± SEM of 4 independent experiments in all experiments performed with 4 replications. *P* values <0.05 were taken to indicate significance.

Results

Cell growth

In comparison to the free Gln group, cell viability was not affected by Ala-Gln ($P > 0.05$), but was significantly reduced by Gly-Gln treatment ($P < 0.05$) (Fig. 1a). The results of 5′-ethynyl-2′-deoxyuridine (EdU) incorporation showed that the percentages of EdU-positive cells were higher ($P < 0.05$) in Gln and Ala-Gln treatment than Gly-Gln treatment (Fig. 1b). The cell proliferation was also investigated by cell cycle analysis that showed the Gly-Gln treatment significantly ($P < 0.05$) increased the percentage of cells in G1 phase, but decreased the percentage of cells in S phase compared with other two treatments (Fig. 2).

Mitochondrial function

The oxygen consumption rate (OCR) is shown in Fig. 3a. And as shown in Fig. 3b, Ala-Gln treatment increased the basal respiration and adenosine triphosphate (ATP) production compared with free Gln treatment, and increased

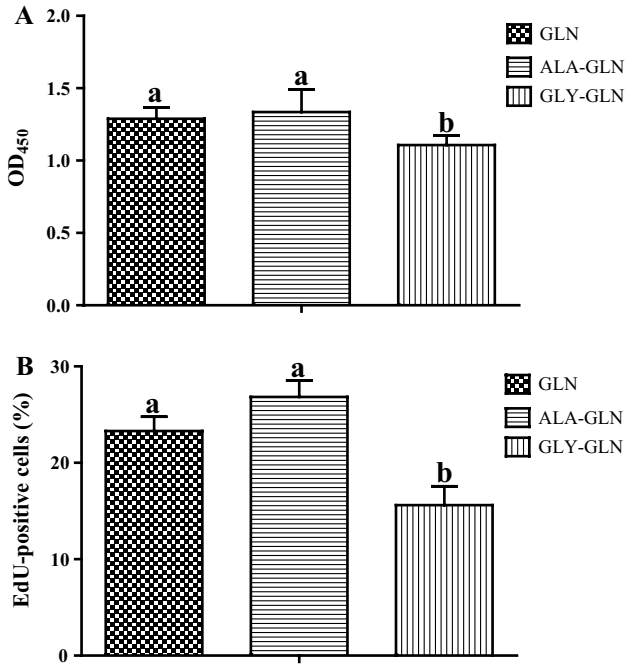

Fig. 1 Cell viability and DNA synthesis of IPEC-J2 cells treated with free Gln, Ala-Gln, and Gly-Gln. Cell viability (**a**) was determined using the Cell Counting Kit-8 (CCK-8, Dojindo Molecular Technologies, Inc, Rockville, MD, USA) at 450 nm. DNA synthesis (**b**) was quantified by EdU incorporation using Cell-Light^{TM} EdU Kit (Rui Bo Biotechnology Limited Company, Guangzhou, China) and presented as the percentage of EdU-positive cells (the number of red nuclei versus the number of blue nuclei in at least five different microscopic fields of vision). Data are expressed as mean ± SEM, $n = 4$ independent experiments. *a*, *b* Means sharing *different letters* differ ($p < 0.05$)

all individual parameters for basal respiration, proton leak, maximal respiration, spare respiratory capacity, non-mitochondrial respiration and ATP production compared with Gly-Gln treatment ($P < 0.05$).

Protein turnover

The protein synthesis of free Gln and Ala-Gln-treated cells were significantly ($P < 0.05$) higher, but the protein degradations were lower than that of Gly-Gln-treated cells. No significant difference about protein synthesis and degradation was observed between free Gln and Ala-Gln group ($P > 0.05$) (Fig. 4).

The relative mRNA expressions of peptide transporter

Significantly lower mRNA expressions of transcription factor specificity protein (Sp1) were observed in Gly-Gln-treated cells than that of free Gln and Ala-Gln treatment ($P < 0.05$). Ala-Gln treatment significantly increased the expression of PepT1 mRNA compared with other two treatments ($P < 0.05$) (Fig. 5).

The relative protein expressions of protein synthesis and degradation pathway

The mammalian target of rapamycin (mTOR) and phosphorylated mTOR protein in the cells treated with free Gln and Ala-Gln present significantly ($P < 0.05$) higher expressions than that of Gly-Gln treatment. Ala-Gln treatment significantly ($P < 0.05$) increased the protein expressions of phosphorylated 4E binding protein-1 (4EBP1), ribosomal protein S6 kinase-1 (S6K1) and phosphorylated S6K1 compared with other two treatments. For the ubiquitin (UB) protein, higher expression in Gly-Gln-treated cells was observed than that of free Gln and Ala-Gln treatment ($P < 0.05$) (Fig. 6).

Discussion

Gln is an essential precursor for synthesizing amino sugars, a major fuel for proliferation and differentiation of intestinal epithelial cells (Braga-Neto et al. 2012; Jiang et al. 2009; Li et al. 2003; Zhou et al. 2012). As an alternative for Gln supplementation, Ala-Gln showed the similar effects on the growth of enterocytes in the present study. Supplementation of Ala-Gln as parenteral nutrition was previously reported to increase mucosal height in the small intestine of rat (Naka et al. 1996). The weight of the intestines from in the early weaned calves parenterally was significantly increased by supplementation with Ala-Gln (Zhou et al. 2012). It was also reported that Ala-Gln could increase mouse intestinal villus length, villus area, crypt depth and cell proliferation and cell migration in vivo (Braga-Neto et al. 2012; Haynes et al. 2009). In addition, following TcdA (a toxin) treatment, Ala-Gln supplementation increased cell proliferation by 137.5% at 24 h and decreased cell apoptosis by 61.4% by increasing RhoA expression to reduced intestinal epithelial cell damage (Santos et al. 2013). However, Gly-Gln treatment showed lower percentages of EdU-positive cells and cells in S phase in this study. Unlike these results, in the jejunal tissues from weaned piglet, the Gly-Gln was reported to improve the cell proliferation and suppress cell apoptosis (Wang et al. 2011). That could be due to the different model and Gly-Gln dose, that 20–30 mmol/L Gly-Gln showed the best effect on the cell proliferation of jejunal tissues by enhancing the glutaminase and diamine oxidase activity, thereby increasing the consumption of glutamine (Wang et al. 2011). This is also probably why Ala-Gln and Gly-Gln have different effects on the proliferation of IPEC-J2.

Whether the Gly-Gln can be utilized by intestinal porcine epithelial cells in vitro is debatable. And significantly lower mRNA expression of Sp1 and PepT1 was also observed in

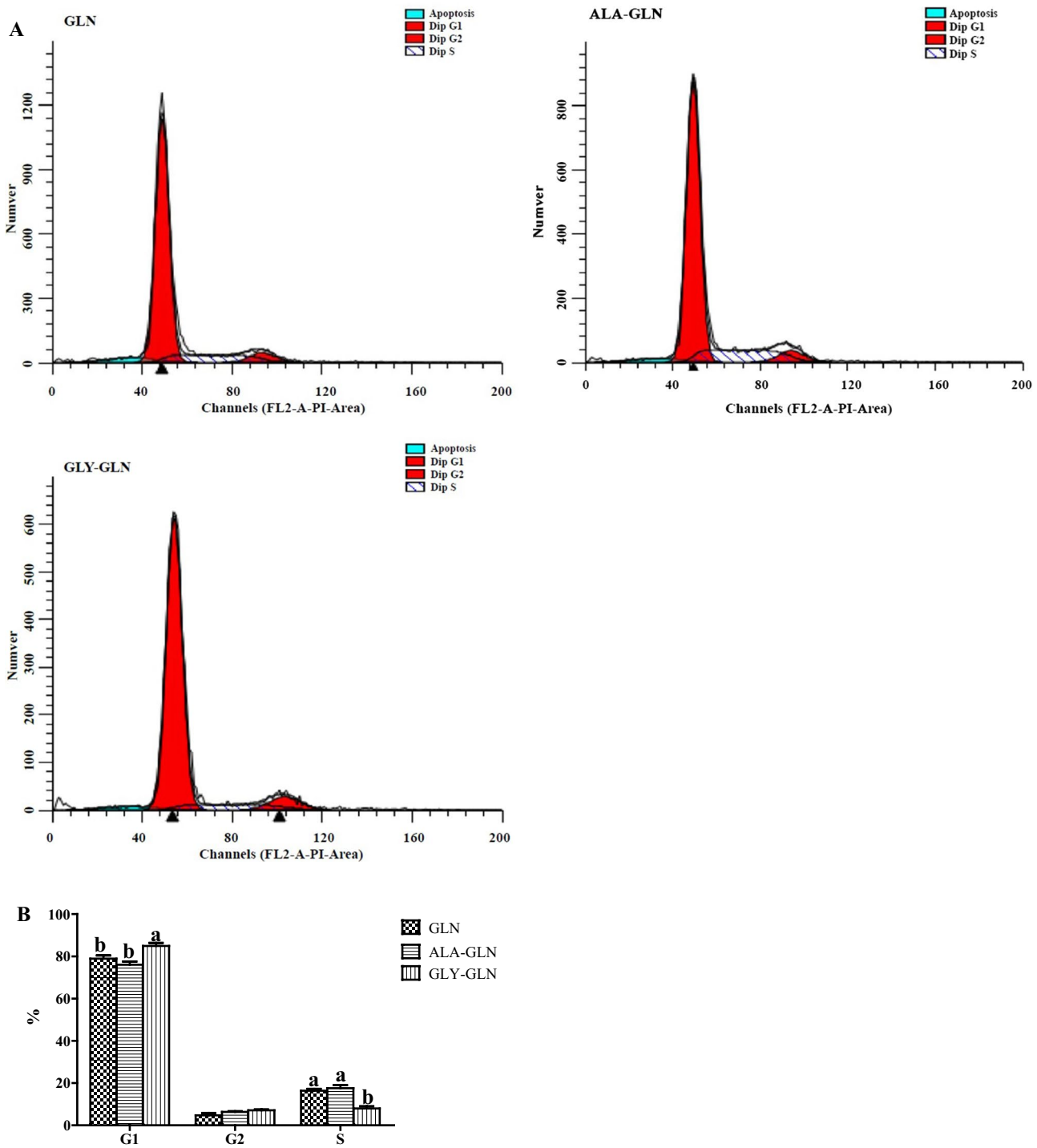

Fig. 2 Cell cycle of IPEC-J2 cells treated with free Gln, Ala-Gln, and Gly-Gln analyzed using propidium iodide DNA staining and Flow Cytometry. **a** Representative flow cytometry diagrams. **b** The percentage of cell population in each phase of the cell cycle. Data are expressed as mean ± SEM, $n = 4$ independent experiments. *a*, *b* Means sharing *different letters* differ ($p < 0.05$)

Gly-Gln-treated cells than that of Ala-Gln. The Gln-containing dipeptides or tripeptides in the lumen of the small intestine can be directly transported into the enterocytes through their apical membrane by H^+ gradient driven PepT1 (Wu et al. 2011). Transport of AA in the form of peptides was demonstrated to be more readily available for absorption than their constituent AA in the free form (Gilbert et al. 2008). But the free Gln had no the same effect as Ala-Gln

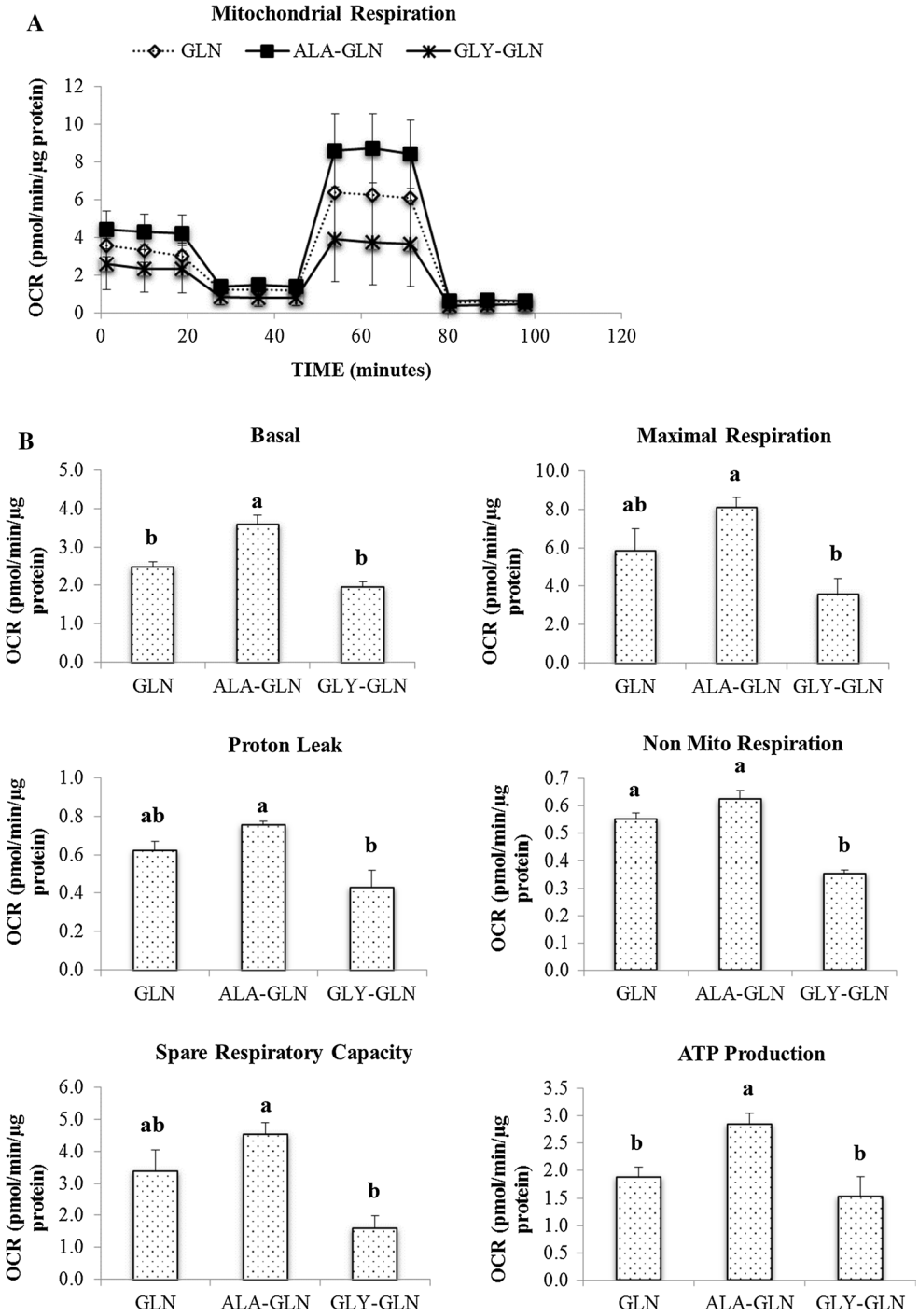

Fig. 3 Mitochondrial respiration of IPEC-J2 cells treated with free Gln, Ala-Gln, and Gly-Gln. Oxygen consumption rate (OCR) (**a**) and individual parameters (basal respiration, proton leak, maximal respiration, spare respiratory capacity, non-mitochondrial respiration and ATP production) (**b**) were assessed by the XF-24 Extracellular Flux Analyzer and Cell Mito Stress Test Kit from Seahorse Biosciences. Data are expressed as mean ± SEM, $n = 4$ independent experiments. *a*, *b* Means sharing *different letters* differ ($p < 0.05$)

on PepT1 activation, this maybe because that PepT1 does not transport free Gln (Wu et al. 2011). Unlike PepT1, the mRNA expression of Sp1, in Gln and Ala-Gln treatment are similar and significantly higher than that of Gly-Gln treatment. Shimakura et al. reported that Sp1 could regulate the transcriptional activity of PepT1 mRNA by acting with the GC-rich sites of PepT1 promoter region (Shimakura et al. 2005). But Sp1 is not the only protein acting through GC-rich sites (Shimakura et al. 2005), which maybe result in that the high level of Sp1 mRNA did not correspondingly present the high expression of PepT1 mRNA in free Gln treatment.

Gln is an important mitochondrial substrate that provide the energy necessary for cell growth and biological activities (Matés et al. 2009). Especially in stress state, a higher O_2 consumption rate and glutathione (GSH) content was observed in Gln treatment. In hyperoxia, Gln protected cellular structures, especially mitochondria, from damage (Ahmad et al. 2001). It also has demonstrated that Gln

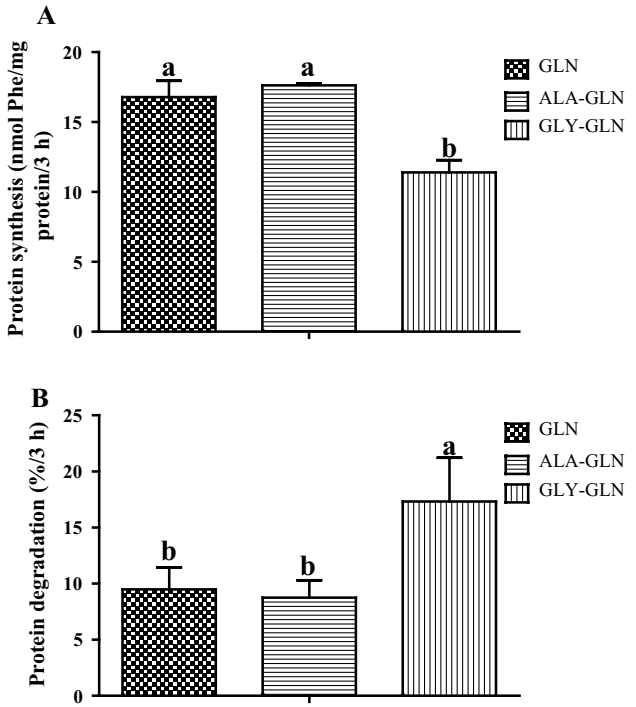

Fig. 4 The protein synthesis (nmol Phe/mg protein/3 h) (**a**) and protein degradation (%) (**b**) of IPEC-J2 cells treated with free Gln, Ala–Gln, and Gly–Gln measured using [^{3}H] labeled phenylalanine. Data are expressed as mean ± SEM, $n = 4$ independent experiments. *a*, *b* Means sharing *different letters* differ ($p < 0.05$)

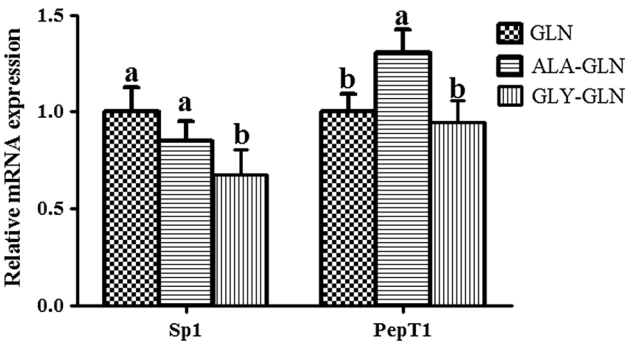

Fig. 5 The relative mRNA expressions of Sp1 and PepT1 in IPEC-J2 cells treated with free Gln, Ala-Gln, and Gly-Gln determined by real-time quantitative RT-PCR. The comparative Ct value method was employed to quantitative expression levels for target genes relative to those for the β-actin. Data are expressed as the relative values to those of free Gln-treated cells and as mean ± SEM, $n = 4$ independent experiments. *a*, *b* Means sharing *different letters* differ ($p < 0.05$)

promoted the repair of intestinal mucosa by providing with fuel and precursors for defense systems (Lallès et al. 2004). In agreement with the results of cell growth, Ala-Gln treatment showed higher basal respiration and ATP production, suggesting the Ala-Gln could be used as oxidizable substrated ATP synthesis in IPEC-J2 more profitably.

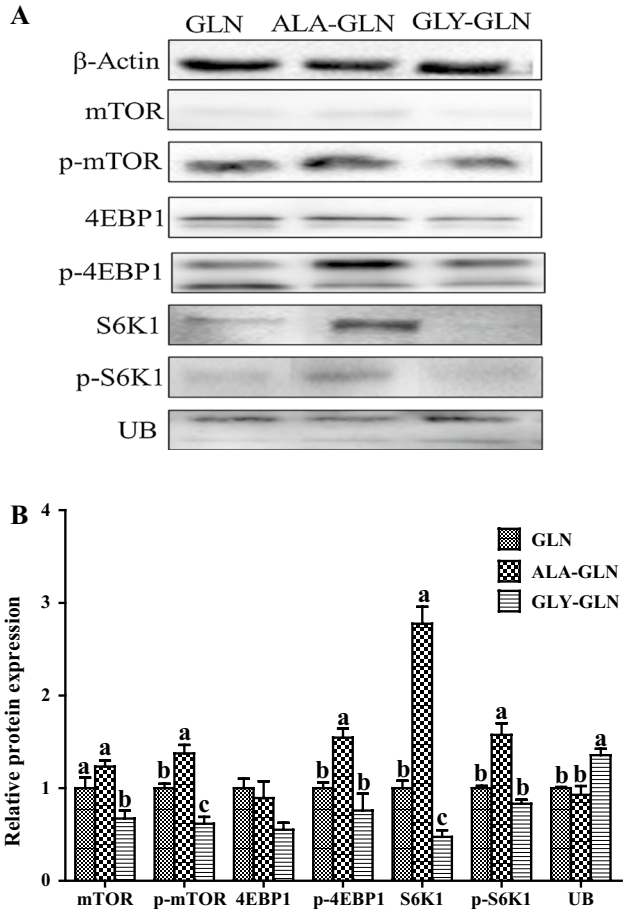

Fig. 6 The representative western blot images (**a**) and relative protein expressions (**b**) of mTOR, 4EBP1, S6K1 and UB in IPEC-J2 cells treated with free Gln, Ala-Gln, and Gly-Gln determined by western blot analysis. All protein measurements were normalized to β-actin. Data are expressed as the relative values to those of free Gln-treated cells and as mean ± SEM, $n = 4$ independent experiments. *a*, *b* Means sharing *different letters* differ ($p < 0.05$)

A number of studies have reported that dietary supplementation with free Gln or Gln dipeptides could significantly enhance the protein synthesis of intestinal porcine epithelial cells (Li et al. 2003; Naka et al. 1996; Wang et al. 2011). Studies on supplementation with other AA, such as leucine and arginine, were also reported to stimulate protein synthesis and inhibit protein degradation (Tan et al. 2010; Yin et al. 2010). Consistent with the cell growth, lower protein synthesis and higher protein degradation in Gly-Gln-treated cells were observed, and the cell signaling pathways (mTOR pathway, ubiquitin proteasome system) that regulate the machinery of intracellular protein turnover were also involved. Gln plays a required role in the uptake of essential amino acids and in maintaining activation of TOR kinase (Wise and Thompson 2010). And the predominant role of the intestinal peptide transporter for the delivery of bulk quantities of amino acids and consequent effects on

insulin and TOR signaling was also reported by Meissner et al. (2004). Therefore, with the increasing in PepT1 mRNA expression, mTOR signaling pathway was activated by Ala-Gln treatment. For protein degradation, the majority of intracellular proteins are degraded by the ubiquitin–proteasome pathway in all tissues (Lecker et al. 2006). And Gln is one of strongest inhibitors of protein degradation in isolated rat hepatocytes (Seglen et al. 1980). Higher protein degradation rate is concomitant with higher UB protein expression in Gly-Gln-treated cells than that of free Gln and Ala-Gln treatment.

In conclusion, this study demonstrated that the dipeptide Ala-Gln had the biological effects to be similar to free Gln as regarding their effects on proliferation, mitochondrial respiration protein turnover in the porcine intestinal cells. These results indicate that Ala-Gln can be substituted Gln as energy and protein sources in the gastrointestinal tract. In contrast, Gly-Gln showed significantly different responses compared to free Gln. The transport activity and metabolism of Ala-Gln and Gly-Gln in IPEC-J2 is needed to study in furture.

Acknowledgements This study was in part supported by the National Natural Science Foundation of China (Nos. 31330075, 31372326, 31672433, 31301989 and 31560640), Key Programs of frontier scientific research of the Chinese Academy of Sciences (QYZDY-SSW-SMC008) and National Basic Research Program of China (2013CB127302). We thank Changsha Lvye Biotechnology Limited Company Academician Expert Workstation, Guangdong Wangda Group Academician Workstation for Clean Feed Technology Research and Development in Swine, Guangdong Hinapharm Group Academician Workstation for Biological Feed and Feed Additives and Animal Intestinal Health for providing technical assistance.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent All authors listed have contributed to conception, design, gathering, analysis or interpretation of data and have contributed to the writing and intellectual content of the article. All authors gave informed consent to the submission of this manuscript.

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