Amino Acids (2014) 46:2037–2045
DOI 10.1007/s00726-014-1758-3

ORIGINAL ARTICLE

Glycine is a nutritionally essential amino acid for maximal growth of milk-fed young pigs

Weiwei Wang · Zhaolai Dai · Zhenlong Wu · Gang Lin · Sichao Jia · Shengdi Hu · Sudath Dahanayaka · Guoyao Wu

Received: 20 April 2014 / Accepted: 25 April 2014 / Published online: 24 May 2014

Abstract Analysis of amino acids in milk protein reveals a relatively low content of glycine. This study was conducted with young pigs to test the hypothesis that milk-fed neonates require dietary glycine supplementation for maximal growth. Fourteen-day-old piglets were allotted randomly into one of four treatments (15 piglets/treatment), representing supplementation with 0, 0.5, 1 or 2 % glycine (dry matter basis) to a liquid milk replacer. Food was provided to piglets every 8 h (3 times/day) for 2 weeks. Milk intake (32.0–32.5 g dry matter/kg body weight per day) did not differ between control and glycine-supplemented piglets. Compared with control piglets, dietary supplementation with 0.5, 1 and 2 % glycine increased ($P < 0.05$) plasma concentrations of glycine and serine, daily weight gain, and body weight without affecting body composition, while reducing plasma concentrations of ammonia, urea, and glutamine, in a dose-dependent manner. Dietary supplementation with 0.5, 1 and 2 % glycine enhanced ($P < 0.05$) small-intestinal villus height, glycine transport (measured using Ussing chambers), mRNA levels for GLYT1, and anti-oxidative capacity (indicated by increased concentrations of reduced glutathione and a decreased ratio of oxidized glutathione to reduced glutathione). These novel results indicate, for the first time, that glycine is a nutritionally essential amino acid for maximal protein accretion in milk-fed piglets. The findings not only enhance understanding of protein nutrition, but also have important implications for designing improved formulas to feed human infants, particularly low birth weight and preterm infants.

Keywords Glycine · Milk replacer · Growth · Intestinal morphology · Piglet

Abbreviations

DM	Dry matter
GSH	Reduced glutathione
GSSG	Oxidized glutathione
KHB	Krebs–Henseleit bicarbonate
OPA	*o*-Phthaldialdehyde

Introduction

Amino acids had long been classified as nutritionally essential or nonessential based on growth or nitrogen balance of animals (see Wu 2013a for review). Nutritionally essential amino acids are those amino acids whose carbon skeletons are not synthesized de novo or those amino acids that usually are not synthesized in adequate amounts to meet the animal's needs and, therefore, must be provided in diets to sustain life and support maximal growth. In contrast, amino acids that are synthesized de novo in animal cells were thought to be dispensable in diets and, therefore, were considered to be nutritionally nonessential; this category of amino acids includes glycine in mammals (Wu 2013a).

Milk was traditionally thought to provide adequate amounts of all amino acids to mammalian neonates (Cunha 1977). However, we have recently reported that sow's milk provides at most only 40 % of arginine for protein accretion in 7- to 21-day-old suckling pigs and that arginine

W. Wang · Z. Dai (✉) · Z. Wu · G. Lin · G. Wu
State Key Laboratory of Animal Nutrition, China Agricultural University, Beijing 100193, China
e-mail: daizhaolai@cau.edu.cn

W. Wang · G. Lin · S. Jia · S. Hu · S. Dahanayaka · G. Wu (✉)
Department of Animal Science, Texas A&M University, College Station, TX 77843, USA
e-mail: g-wu@tamu.edu

deficiency is a major factor limiting their maximum growth (Kim and Wu 2004; Wu and Knabe 1994). Based on glycine content in sow's milk and the accretion of glycine in the whole body, we have estimated that the milk meets at most 23 % of daily glycine needs for protein synthesis in the young pig and that the neonate must synthesize at least 0.71 g glycine/kg body weight per day (Wu 2010). The high requirement for glycine fulfills its multiple physiological functions in the body (Kawai et al. 2012; Schemmer et al. 2013; Wang et al. 2013; Wu 2009).

Based on the foregoing, we hypothesized that milk-fed neonates require dietary glycine supplementation for maximal growth. This hypothesis was tested in the present study involving young pigs fed a liquid milk replacer diet supplemented with 0, 0.5, 1 or 2 % glycine [dry matter (DM) basis]. Our results unequivocally demonstrate, for the first time, that glycine is a nutritionally essential amino acid for maximal growth of milk-fed young pigs.

Materials and methods

Materials

HPLC-grade water and methanol were obtained from Fisher Scientific (Houston, TX, USA). L-Arginine-HCl and L-alanine were obtained from Ajinomoto Inc. (Tokyo, Japan). Glycine and cornstarch were procured from Sigma Chemicals (St. Louis, MO, USA).

Milk replacer diet

Milk replacer powder, which consisted of dried bovine whey protein concentrate, dried whey, vegetable and animal fat, and lactose (Kim and Wu 2004), was used for this study. Glycine content in the milk replacer powder was 5.65 g/kg DM, as analyzed using acidic analysis and high-performance liquid chromatography (Dai et al. 2014). The liquid diet was prepared by mixing 1 kg of milk replacer powder (96.7 % DM) with 4.2 L of water to obtain milk solution (18.6 % DM), which matched the DM content (18.6 %) of sow's milk on days 14–28 of lactation (Rezaei et al. 2011). This basal diet was supplemented with 0.48 % L-arginine-HCl, providing 0.4 % L-arginine to meet requirements for milk-fed piglets (Kim and Wu 2004).

Animals and experimental design

Piglets

The experimental protocol of this study was approved by the Texas A&M University Institutional Animal Care and Use Committee.

Table 1 Addition of glycine or L-alanine to milk replacer powder

Treatment	0 % Gly	0.5 % Gly	1 % Gly	2 % Gly
Glycine	0	5	10	20
L-Alanine	23.7	17.8	11.9	0
Cornstarch	0	0.9	1.8	3.7

The milk replacer powder contained 96.7 % DM

Values are g/kg DM. Glycine: MW = 75.1. L-Alanine: MW = 89.1

Piglets were offspring of Yorkshire × Landrace sows and Duroc × Hampshire boars and were maintained at the Texas A&M University Swine Center. At 11 days of age, piglets were removed from sows and housed in a nursery room (32 °C, relative humidity of 60 %). Piglets were fed every 8 h (3 times/day) the basal liquid milk (prewarmed to 35 °C) at 150, 165 and 180 mL/kg body weight per day at 11, 12 and 13 days of age, respectively.

Dietary supplementation with glycine to milk-fed piglets

At 14 days of age, piglets were allotted randomly to one of four treatment groups on the basis of body weight and litter. Each treatment group consisted of 5 pens (3 piglets/pen). Piglets in Groups 1, 2, 3 and 4 received dietary supplementation with 0, 0.5, 1 or 2 % glycine (DM basis), respectively, to the basal liquid milk replacer. In addition, appropriate amounts of L-alanine and cornstarch were added to the milk replacer to formulate isonitrogenous and isocaloric diets (Table 1). All piglets were provided with their respective diets (prewarmed to 35 °C) every 8 h (3 times/day) at 180 mL/kg body weight/day between 14 and 28 days of age. Actual food consumption was measured daily, and feeders were cleaned with water daily. Pair-feeding was used to ensure similar intakes of all dietary nutrients, except for glycine. The temperature of the nursery facility was 29 °C and 27 °C during the third and fourth weeks after birth, respectively, and its relative humidity was 60 %. At 14, 21 and 28 days of age, piglets were weighed individually before the first feeding.

Blood sampling for analysis of amino acids, ammonia, urea and glutathione in plasma

At 21 and 28 days of age, jugular venous blood samples (4 mL) were obtained from each piglet at 1 h after feeding. Amino acids and glutathione in plasma were analyzed by HPLC methods, as previously described (Rezaei et al. 2013; Wu and Meininger 2008; Wu et al. 1997). Ammonia and urea in plasma were determined using enzymatic methods (Rezaei et al. 2013; Satterfield et al. 2012).

Collection of the small intestine from pigs

At 28 days of age, 2 h after blood sampling, pigs were anesthetized with intramuscular injection of Telazol (10 mg/kg body weight) and then euthanized by intracardial administration of saturated KCl. After the abdomen was opened, the duodenum (10 cm from the pyloric junction), jejunum (proximal half of the remaining small intestine), and ileum (15 cm before the ileo-caecal junction) were quickly isolated (He et al. 2013a). Luminal contents in the duodenum, jejunum and ileum were obtained for analysis of free amino acids (Wu and Meininger 2008) and then the small-intestinal lumen was flushed three times with ice-cold saline (Wang et al. 2008). Portions of the duodenum, jejunum, and ileum were frozen rapidly in liquid nitrogen for subsequent analysis of reduced glutathione (GSH) and oxidized glutathione (GSSG), as we described previously (Rezaei et al. 2013). Furthermore, each segment (1 cm) of the small intestine was immediately fixed in 4 % paraformaldehyde and then embedded in paraffin for analysis of intestinal morphology (Wu et al. 1994).

Measurement of amino acid transport by the jejunum using Ussing chambers

Transport of glycine or glutamine by the small intestine depends on sodium (Wu et al. 2014) and, therefore, is associated with changes in short circuit current across the tissue. For the measurement of intestinal glycine, glutamine, glutamate and arginine transport by Ussing chambers (VCC MC6, Physiologic Instruments, San Diego, CA), a segment of freshly isolated jejunum (~10 cm) was placed in 20 mL of the Krebs–Henseleit bicarbonate (KHB) buffer (37 °C, pH 7.4; gassed with 95 % O_2/5 % CO_2) containing 5 mM D-glucose and 20 mM HEPES (pH 7.4) (Wu et al. 1995). A jejunum segment (1 cm^2) was mounted onto a slider (Cat. # P2305, Physiologic Instruments), which was then inserted into the Ussing chamber (Ducroc et al. 2010). The KHB buffer (5 mL) was placed into each side of the Ussing chamber and was continuously gassed with 95 % O_2/5 % CO_2. The temperature of this solution was maintained at 37 °C through a circulatory water bath set at 41 °C. Transport of an amino acid (1 and 5 mM) was initiated by its addition to the mucosal side of the Ussing chamber. Electrogenic ion transport induced by amino acid uptake into the intestinal mucosa was monitored continuously as changes in short circuit current (He et al. 2013b). Results were expressed as the percent of the short circuit current for the jejunum of the control group without exposure to any added amino acid in the Ussing chambers.

Determination of GSH and GSSG in plasma and small intestine

The analysis of GSSG and GSH is based on the principle that GSSG is reduced by 2-mercaptoethanol to GSH, which reacts with iodoacetic acid to form S-carboxymethyl-glutathione. S-carboxymethyl-glutathione is subsequently derivatized with *o*-phthaldialdehyde (OPA) to form a highly fluorescent adduct. A frozen intestinal tissue (~100 mg) was homogenized with 1.5 mL of homogenization buffer (a mixture of 12 mM iodoacetic acid and 1.5 M $HClO_4$; 50:50, v/v), and the homogenate was transferred to a 15-mL polystyrene tube. The homogenizer was rinsed with 1.5 mL of the homogenization buffer. The combined homogenate was neutralized with 0.75 mL of 2 M K_2CO_3. The tube was centrifuged at 600×*g* for 5 min, and the neutralized supernatant fluid was used for the analysis of GSG and GSSG. Specifically, for analysis of GSH, a 4-mL glass vial contained 50 μL of 100 μM GSH standard or neutralized sample and 100 μL of 40 mM sodium borate. For analysis of total glutathione (GSH + GSSG), a 4-mL glass vial contained 50 μL of 50 μM GSSG standard or sample and 100 μL of 28 mM 2-mercaptoethanol (prepared in 40 mM sodium borate). To both the GSH and total glutathione assay vials, 50 μL of 25 mM iodoacetic acid, 0.1 mL of 1.2 % benzoic acid (prepared in saturated $K_2B_4O_7$), and 1.4 mL of HPLC H_2O were added sequentially. The vials were placed in an autosampler where 15 μL of the solution was programmed to mix with 15 μL of 14.5 mM OPA for 1 min and the derivatized product was injected, without any delay, into a Supelco C_{18} guard column (4.6 mm × 5 cm, 20–40 μm) connected to a Supelco C_{18} analytical column (4.6 mm × 15 cm, 3 μm). GSH was eluted from the column using a 16-min gradient program (including the time for column regeneration) that involved mobile phase A (0.1 M sodium acetate, pH 7.2; consisting of 27.3 g of sodium acetate-trihydrate, 1.81 L H_2O, 96 μL of 6 M HCl, 180 mL HPLC-grade methanol, and 10 mL tetrahydrofuran) and mobile phase B (HPLC-grade methanol). The solvent gradient for mobile phase A was: 0–1 min, 97 %; 1.1–6.5 min, 86 %; 6.6–9.0 min, 0 %; 9.1–16 min, 97 %. The GSH–OPA derivative was detected (retention time = 7.0 min) using a Waters Model 2475 Multi λ Fluorescence Detector at excitation wavelength 220 nm between 0 and 6 min and also between 12 and 16 min, at excitation wavelength 340 nm between 6 and 12 min, and at emission wavelength 450 nm between 0 and 16 min. The setting of excitation wavelength at 220 nm before 6 min and after 12 min was designed to suppress fluorescence due to amino acids that directly react with OPA. The amount of GSSG was calculated as (total glutathione − GSH)/2.

Table 2 Primers for genes encoding amino acid transporters and Na^+/K^+-ATPase in the small intestine

Gene	Protein	Substrates	System	Primer sequences (5′–3′)	
SLC6A19	B^0AT1	All neutral amino acids	NBB (B^o)	Forward	CACAACAACTGCGAGAAGGA
				Reverse	CCGTTGATAAGCGTCAGGAT
SLC1A3	EAAT1	Glu, Asp	X_{AG}	Forward	GATGGGACCGCCCTCTAT
				Reverse	CGTGGCTGTGATGCTGATG
SLC1A5	ASCT2	Ala, Ser, Cys, Thr, Gln	ASC	Forward	GCCAGCAAGATTGTGGAGAT
				Reverse	GAGCTGGATGAGGTTCCAAA
SLC7A1	CAT-1	Arg, His, Lys, Orn	y^+	Forward	TGCCCATACTTCCCGTCC
				Reverse	GGTCCAGGTTACCGTCAG
SLC7A7	y^+LAT1 (4F2hc)	Arg, Lys, His, Gln, Leu, Ala, Cys, Met	y^+L	Forward	GCCCATTGTCACCATCATC
				Reverse	GAGCCCACAAAGAAAAGC
SLC3A1	rBAT	Cystine, basic and neutral amino acids	HC-HAAT	Forward	TTTCCGCAATCCTGATGTTC
				Reverse	GGGTCTTATTCACTTGGGTC
SLC6A9	GLYT1	Gly, sarcosine	Gly	Forward	CCATGTTCAAAGGAGTGGGCTA
				Reverse	TGACCACATTGTAGTAGATGCCG
SLC7A9	b^{0+}AT (rBAT)	Arg, Lys, Orn, Cystine	b^{0+}	Forward	ATCGGTCTGGCGTTTTAT
				Reverse	GGATGTAGCACCCTGTCA
SLC38A1	SNAT1	Gln, Ala, Cys, Asn, His, Gly, Met	A	Forward	CAGCCGCTCTCAACAGTCT
				Reverse	AGTTCCGCAGTATCAGTGGT
SLC38A3	SNAT3	Gln, His, Asn, Cit	N	Forward	GACCCCAACACCCACATGAT
				Reverse	GCCCTAGCACACCTCAGTTT
SLC6A5	GLYT2	Gly, sarcosine	Gly	Forward	CGCCCTTGGTAACTTCCACT
				Reverse	CTGTGCAGTTTGATGGGCAC
ATP1A1	ATPase, Na^+/K^+ transporting, α1 polypeptide			Forward	ATCGCAAATACGGAACGGACT
				Reverse	GCCGACAGAACTTGACCCAT
β-Actin	Housekeeping gene (reference gene)			Forward	TGCGGGACATCAAGGAGAAG
				Reverse	AGTTGAAGGTGGTCTCGTGG

For the analysis of GSH and GSSG in plasma, whole blood sample (0.2 mL) was gently mixed with 0.2 mL of Solution A (preservation solution; consisting of 1 mg sodium heparin, 10 mg L-serine, 4.5 mg iodoacetic acid, and 2 mL of 100 mM sodium borate) in a 1.5-mL tube. The tube was centrifuged at 10,000×*g* for 1 min. The supernatant fluid (0.2 mL) was added to a new 1.5-mL tube, to which 0.1 mL of Solution B (1.5 M $HClO_4$ and 0.2 M boric acid; consisting of 1.24 g boric acid, 87.1 mL of HPLC-grade H_2O, and 12.9 mL of 70 % $HClO_4$) and 50 μL of 2 M K_2CO_3 were added sequentially. The tube was centrifuged at 10,000×*g* for 1 min, and the neutralized supernatant fluid was used for the analysis of GSH and total glutathione, as described previously.

Determination of mRNA levels for amino acid transporters and ATPase

Total RNA was extracted from each jejunal sample using the RNeasy Mini Kit (QIAGEN) and reverse transcribed to determine mRNA levels for amino acid transporters and ATPase, as previously described (Jobgen et al. 2009; Yin et al. 2014; Zhang et al. 2013). β-Actin was used as a housekeeping gene, as its mRNA level was not affected by the dietary treatment (Wang et al. 2010). Primer sequences for select amino acid transporters and β-actin are summarized in Table 2. Quantitative PCR was performed with SYBR Green and the thermal cycler conditions for PCR were 95 °C for 5 min followed by 34 cycles at 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s, and then at 72 °C for 10 min. Data analysis was performed, as described by Fu et al. (2010).

Determination of body composition

The whole body of each pig without its intestinal contents was homogenized using a Seydelmann Cutter K64 (Strasser; Stuttgart, Germany), as described by Satterfield et al. (2012). The content of DM, crude protein, lipids, carbohydrate, and ash (minerals) was determined by the standard methods (Wu et al. 1999).

Statistical analysis

Values are presented as mean ± SEM. Data were analyzed by one-way ANOVA using the SAS Statistical Software (SAS Institute Inc., NC, USA). Differences among treatment means were determined using the Student–Newman–Keuls multiple comparison test (Fu et al. 2010). Probability values ≤0.05 were taken to indicate statistical significance (Wei et al. 2012).

Results

Milk intake and growth performance

Compared with the control group, food intake per kg body weight did not differ among the treatment groups (Table 3) due to the pair-feeding procedure used in the present study. Compared with control piglets, dietary supplementation with 0.5, 1 and 2 % glycine increased ($P < 0.05$) daily weight gain over the 14-day experimental period and the body weight of piglets at 14 and 28 days of age, in a dose-dependent manner (Table 3).

Plasma amino acids, ammonia, urea, and glutathione

Compared with the control group, dietary supplementation with 0.5, 1 and 2 % increased ($P < 0.05$) plasma concentrations of glycine and serine, but reduced plasma concentrations of glutamine, ammonia and urea, in a dose-dependent manner (Table 4). Plasma concentrations of all other amino acids (including arginine, histidine, lysine, ornithine, aspartate, glutamate, alanine, proline, and hydroxyproline) did not differ among the four groups of piglets (data not shown). Dietary supplementation with 1 and 2 % glycine increased ($P < 0.05$) concentrations of GSH in plasma, compared with the control group (Table 4). Dietary supplementation with 1 and 2 % glycine dose-dependently decreased ($P < 0.05$) the ratio to GSSG to GSH in plasma (Table 4).

Table 3 Effects of dietary supplementation with glycine on growth performance in milk-fed piglets

Items	Glycine supplementation (%)				Pooled SEM	*P* value
	0	0.5	1	2		
Body weight (kg)						
Day 0	3.68	3.64	3.69	3.63	0.10	0.986
Day 7	5.27^{c}	5.41^{bc}	5.62^{ab}	5.82^{a}	0.13	0.012
Day 14	7.05^{c}	7.33^{bc}	7.71^{ab}	8.06^{a}	0.15	<0.001
Average daily weight gain (g/day)						
Days 0–7	227^{d}	253^{c}	276^{b}	313^{a}	6.4	<0.001
Days 7–14	248^{d}	274^{c}	298^{b}	320^{a}	7.1	<0.001
Days 0–14	241^{d}	264^{c}	288^{b}	316^{a}	6.2	<0.001
Average daily food intake (g DM/kg BW/day)						
Days 0–7	32.3	32.0	32.5	32.2	1.4	0.990
Days 7–14	32.5	32.2	32.3	32.4	1.2	0.965
Days 0–14	32.2	32.1	32.0	32.0	1.1	0.944

Values are means with pooled SEM, $n = 15$ for body weight and daily weight gain, and $n = 5$ for food intake. Day 0 = 14 days of age

[a–d] Within a row, means sharing different superscript letters differ ($P < 0.05$)

Table 4 Effects of dietary supplementation with glycine on plasma concentrations of amino acids, ammonia, urea and glutathione in milk-fed piglets at 21 and 28 days of age

Amino acids	Glycine supplementation (%)				Pooled SEM	*P* value
	0	0.5	1	2		
21-day-old pigs						
Glycine	1016^{d}	1748^{c}	2205^{b}	2941^{a}	74	<0.001
Serine	201^{d}	265^{c}	302^{b}	344^{a}	8.3	<0.001
Glutamine	628^{a}	581^{b}	540^{c}	503^{d}	7.0	<0.001
Ammonia	75^{a}	63^{b}	52^{c}	40^{d}	3.2	<0.001
Urea	2258^{a}	2064^{b}	1865^{c}	1662^{d}	68	<0.001
GSH	4.52^{d}	5.03^{c}	5.67^{b}	6.23^{a}	0.12	0.004
GSSG	0.95^{a}	0.93^{a}	0.84^{b}	0.79^{b}	0.03	0.001
GSSG/GSH[A]	0.212^{a}	0.180^{b}	0.145^{c}	0.124^{d}	0.004	<0.001
28-day-old pigs						
Glycine	1127^{d}	1833^{c}	2294^{b}	2890^{a}	69	<0.001
Serine	214^{d}	249^{c}	277^{b}	318^{a}	7.6	<0.001
Glutamine	636^{a}	592^{b}	551^{c}	510^{d}	6.4	<0.001
Ammonia	73^{a}	61^{b}	50^{c}	41^{d}	2.8	<0.001
Urea	2361^{a}	2175^{b}	1932^{c}	1728^{d}	63	<0.001
GSH	4.86^{d}	5.32^{c}	5.79^{b}	6.42^{a}	0.14	<0.001
GSSG	0.97^{a}	0.95^{a}	0.82^{b}	0.75^{b}	0.02	<0.001
GSSG/GSH[A]	0.201^{a}	0.176^{b}	0.143^{c}	0.116^{d}	0.006	<0.001

Values, expressed as μM, are means with pooled SEM, $n = 15$

[A] Expressed as μmol/μmol

[a–d] Within a row, means sharing different superscript letters differ ($P < 0.05$)

Changes in short circuit current in response to amino acid transport by the jejunum

Compared with the absence of an added amino acid, addition of 1 and 5 mM glycine, L-glutamine L-glutamate or L-arginine to the mucosal side of the Ussing chamber dose-dependently increased ($P < 0.05$) short circuit current in the jejunum from all groups of piglets (Table 5). Dietary supplementation with 0.5, 1 or 2 % glycine had no effect

Table 5 Effects of dietary supplementation with glycine on short circuit current across the jejunum of milk-fed piglets

Addition of amino acid to the Ussing chamber	Glycine supplementation in diet (%)				Pooled SEM	*P* value
	0	0.5	1	2		
Glycine						
0 mM	1.00	1.05	1.03	1.06	0.04	0.962
1 mM	1.26^{d}	1.49^{c}	1.75^{b}	2.13^{a}	0.08	<0.001
5 mM	1.77^{d}	2.09^{c}	2.46^{b}	2.89^{a}	0.10	<0.001
L-Glutamine						
0 mM	1.00	1.03	1.07	1.04	0.05	0.948
1 mM	1.52	1.64	1.58	1.71	0.07	0.975
5 mM	2.38	2.50	2.61	2.55	0.10	0.967
L-Glutamate						
0 mM	1.00	0.96	0.92	1.04	0.04	0.188
1 mM	1.64	1.78	1.62	1.80	0.06	0.086
5 mM	2.45	2.53	2.65	2.68	0.09	0.252
L-Arginine						
0 mM	1.00	1.08	0.96	1.10	0.06	0.194
1 mM	1.21	1.30	1.23	1.36	0.07	0.426
5 mM	1.57	1.66	1.61	1.79	0.07	0.183

Values are means with pooled SEM, $n = 15$. Addition of glycine, glutamine, glutamate or arginine to the mucosal side of the Ussing chamber dose-dependently increased ($P < 0.05$) short circuit current in the jejunum from pigs supplemented with 0, 0.5, 1 or 2 % glycine. The actual short circuit current for the jejunum of the control group without exposure to any added amino acid in Ussing chambers was 27.4 ± 1.3 μA/cm^2 (mean ± SEM, $n = 15$). Dietary supplementation with glycine did not affect L-glutamine, glutamate or arginine transport by the jejunum from any group of pigs. Values are means with pooled SEM, $n = 15$

$^{a–d}$ Within a row, means sharing different superscript letters differ ($P < 0.05$)

on short circuit current in the jejunum exposed to 0, 1 or 5 mM L-glutamine, L-glutamate or L-arginine (Table 5).

Morphology and weight of the small intestine and its glutathione concentrations

Compared with the control group, dietary glycine supplementation dose-dependently enhanced ($P < 0.05$) glycine concentrations in the lumen of the small intestine, as well as villus height in the duodenum, jejunum, and ileum, but did not affect crypt depths in these segments of the small intestine (Table 6). The weight of the entire small intestine was greater ($P < 0.05$) in glycine-supplemented pigs, compared with control pigs (Table 5). In addition, concentrations of GSH were higher ($P < 0.05$), but concentrations of GSSG and the ratio of GSSG to GSH were reduced ($P < 0.05$), in the small intestine of glycine-supplemented pigs, compared with the control group (Table 6).

mRNA levels for select amino acid transporters and ATPase

Effects of dietary supplementation with 0.5, 1 and 2 % glycine increased ($P < 0.05$) jejunal mRNA levels for GLYT1 in a dose-dependent manner (Table 7). Glycine supplementation did not affect mRNA levels for SLC6A19, SLC1A3, SLC1A5, SLC7A1, SLC7A7, SLC3A1, SLC7A9, SLC6A5, SLC38A1, SLC38A3, or Na^+/K^+-ATPase in the jejunum of milk-fed piglets. Thus, expression of the genes encoding the proteins of transport systems for basic and acid amino acids, as well as many neutral amino acids was not influenced by dietary glycine supplementation.

Body composition of piglets

Water represented 30.7 % of body weight in 28-day-old pigs and protein was quantitatively the most abundant component of DM in the piglet body (Table 8). Dietary supplementation with glycine did not affect body composition in young pigs.

Discussion

Glycine was traditionally classified as a nutritionally non-essential amino acid for mammals (Wang et al. 2010; Wu 2009). Thus, few studies have been conducted to determine effects of dietary glycine levels on growth performance and physiological responses in humans or livestock species (Wu et al. 2014). Wu (2010) has suggested that dietary glycine is inadequate for the need of this amino acid by pigs fed a milk protein- or corn- and soybean meal-based diet. In support of this view, Powell et al. (2011) reported that the rate of glycine biosynthesis in 20–50 kg pigs fed a low-protein diet could not meet its requirement for optimal growth performance. Thus, dietary supplementation with 0.52 % glycine to the low-protein diet increased average daily gain and feed efficiency of growing pigs (Powell et al. 2011). Similar results were obtained for young pigs fed a milk-based diet when food intake did not differ among the treatment groups (Table 3). Because the body composition of piglets was not affected by glycine supplementation (Table 8), their weight gains resulted primarily from protein deposition in the body but not a disproportionate increase in fat accumulation.

Multiple pathways are involved in glycine metabolism via inter-organ coordination (Kalhan 2013; Minelli et al. 2012; Wu 2013b). In humans, a large amount (~41 %) of glycine flux contributes to serine biosynthesis through serine hydroxymethyltransferase (Shemin 1950). Results of the present study showed that dietary supplementation with

Table 6 Effects of dietary supplementation with glycine on free glycine concentrations in intestinal luminal fluid, small intestinal morphology, and glutathione concentrations in the small intestine in milk-fed piglets

Items	Glycine supplementation (%)				Pooled SEM	*P* value
	0	0.5	1	2		
Villus height (μm)						
Duodenum	411[d]	426[c]	442[b]	457[a]	5.1	<0.001
Jejunum	469[d]	486[c]	504[b]	522[a]	5.4	<0.001
Ileum	357[d]	372[c]	386[b]	405[a]	4.7	<0.001
Crypt depth (μm)						
Duodenum	262	254	249	257	4.0	0.152
Jejunum	232	239	241	244	3.8	0.164
Ileum	220	216	223	228	3.6	0.133
Weight of the small intestine (g)	226[d]	238[c]	251[b]	263[a]	3.9	<0.001
GSH (μmol/g tissue)						
Duodenum	2.02[d]	2.26[c]	2.59[b]	2.86[a]	0.05	<0.001
Jejunum	2.24[d]	2.50[c]	2.83[b]	3.11[a]	0.06	<0.001
Ileum	2.10[d]	2.37[c]	2.62[b]	2.83[a]	0.05	<0.001
GSSG (nmol/g tissue)						
Duodenum	182[a]	160[b]	143[c]	129[d]	1.8	<0.001
Jejunum	191[a]	174[b]	156[c]	140[d]	2.1	<0.001
Ileum	186[a]	165[b]	147[c]	131[d]	1.9	<0.001
GSSG/GSH (μmol/μmol)						
Duodenum	0.091[a]	0.072[b]	0.056[c]	0.044[d]	0.004	<0.001
Jejunum	0.087[a]	0.070[b]	0.055[c]	0.045[d]	0.003	<0.001
Ileum	0.090[a]	0.068[b]	0.057[c]	0.048[d]	0.003	<0.001
Glycine concentrations in the small-intestinal lumen fluid (mM)						
Duodenum	0.86[d]	2.46[c]	4.81[b]	6.95[a]	0.18	<0.001
Jejunum	2.04[d]	3.28[c]	6.57[b]	9.36[a]	0.22	<0.001
Ileum	1.25[d]	1.64[c]	1.98[b]	2.30[a]	0.05	<0.001

Values are means with pooled SEM, $n = 15$

[a–d] Within a row, means sharing different superscript letters differ ($P < 0.05$)

glycine dramatically increased its concentrations in the plasma of milk-fed piglets (two- to fourfold) (Table 4). This suggests a low rate of the catabolism of dietary glycine by enterocytes of the piglet small intestine during the first pass. However, absolute increases in circulating levels of serine were much lower than those for glycine (Table 4). These results are similar to those reported for adult rats (Shoham et al. 2001). We suggest that: (1) synthesis of serine from glycine is limited in the small intestine; (2) rates of whole-body conversion of glycine into serine are relatively low in young pigs; and (3) most of the glycine in the metabolic free pool (e.g., plasma) is utilized for synthesis of protein and other nitrogenous products (e.g., glutathione). It is likely that neonates efficiently conserve glycine for tissue growth.

A novel and important finding from this study is that dietary supplementation with glycine markedly reduced concentrations of glutamine in the piglet plasma (Table 4). Because glycine had no effect on glutamine transport by the small intestine (Table 5), it is likely that de novo synthesis of glutamine in skeletal muscle and other tissues was decreased in response to glycine supplementation. This could result from a reduced availability of ammonia owing to enhanced protein synthesis and reduced oxidation of amino acids in the whole body. In support of this view, concentrations of both ammonia and urea in the plasma were lower in glycine-supplemented piglets, compared with the control group (Table 4). Therefore, adequate supplementation with glycine to a milk-based diet is necessary for maximal protein accretion and growth of young pigs. Because human milk is also severely deficient in glycine like sow's milk (Davis et al. 1994; Wu and Knabe 1994), its dietary supplementation to formulas (which are generally designed according to amino acid composition in human milk) may improve optimal growth and development of infants, particularly preterm or low birth weight infants (Lin et al. 2014). This may also be of particular importance for neonates nursed by women and other mammals (e.g., sows and cows) who produce inadequate milk during lactation (Lei et al. 2012).

Recent studies indicate the important functions of amino acids in maintaining gut health and development (Wang et al. 2009). The beneficial roles of glycine on protecting intestine are supported by its cytoprotective effect against

Table 7 Effects of dietary supplementation with glycine on mRNA levels for select amino acid transporters and Na^+/K^+-ATPase in the jejunum of milk-fed piglets

Gene	Glycine supplementation in diet (%)				Pooled SEM	*P* value
	0	0.5	1	2		
SLC6A19	1.00	1.05	1.14	1.12	0.06	0.338
SLC1A3	1.00	1.08	1.10	1.15	0.09	0.704
SLC1A5	1.00	0.93	1.02	1.06	0.08	0.720
SLC7A1	1.00	1.08	1.04	1.13	0.09	0.775
SLC7A7	1.00	1.10	1.16	1.09	0.07	0.462
SLC3A1	1.00	1.07	1.12	1.03	0.06	0.534
SLC7A9	1.00	1.12	1.06	1.15	0.08	0.568
SLC6A9	1.00[d]	1.36[c]	1.75[b]	2.28[a]	0.12	<0.001
SLC38A1	1.00	0.92	1.04	0.96	0.14	0.752
SLC38A3	1.00	1.03	0.98	1.07	0.17	0.807
SLC6A5	1.00	0.96	0.93	0.94	0.15	0.796
ATP1A1	1.00	0.97	0.94	1.07	0.07	0.609

mRNA levels for the target gene were normalized to those for β-actin. Values for the control group were taken to be 1.00. Data are means with pooled SEM, $n = 8$

[a–d] Within a row, means sharing different superscript letters differ ($P < 0.05$)

Table 8 Effects of dietary supplementation with glycine on the body composition of 28-day-old piglets fed a milk replacer diet

Items	Glycine supplementation (%)				Pooled SEM	*P* value
	0	0.5	1	2		
Water	69.2	69.3	69.4	69.3	0.7	0.998
DM	30.8	30.7	30.6	30.7	0.7	0.998
Crude protein	15.0	15.1	14.9	15.0	0.5	0.995
Crude fat	12.4	12.3	12.2	12.3	0.4	0.989
Crude CH_2O	0.30	0.31	0.29	0.29	0.02	0.886
Crude ash	3.1	3.0	3.2	3.1	0.1	0.602

Values, expressed as percent of body weight (%), are means with pooled SEM, $n = 10$

CH_2O carbohydrates

intestinal ischemia–reperfusion injury (de Aguiar et al. 2011; Fuchs et al. 2012). Glycine preconditioning can protect mitochondrial viability during intestinal hemorrhages and normalize arterial blood pressure during oxidative injury to the small intestine (Petrat et al. 2011; Wang et al. 2012). In well-established chemical models of colitis, glycine can protect against intestinal injury (Tsune et al. 2003), further highlighting its important role in maintaining intestinal health. This is consistent with our results that dietary supplementation with glycine increased concentrations of GSH and decreased concentrations of GSSG in the piglet small intestine (Table 6), while improving intestinal morphology and enhancing glycine transport by the small intestine (Table 5). Similarly, jejunal mRNA levels for GLY1 were augmented by dietary glycine supplementation (Table 7), indicating a role for glycine in regulating intestinal gene expression. As the uptake of arterial glycine by the small intestine is negligible (Wu et al. 2014) and enteral glycine is required for maximal GSH synthesis in the piglet intestinal mucosa (Reeds et al. 1997), the neonatal gut must obtain adequate glycine from the diet to sustain its structure and function.

In conclusion, dietary supplementation with 0.5, 1 and 2 % glycine increased small-intestinal villus height, intestinal transport of glycine, plasma concentrations of glycine and GSH, as well as whole-body growth and protein accretion, while reducing plasma concentrations of ammonia, urea, and glutamine, in a dose-dependent manner. A milk-based diet plus endogenous synthesis cannot provide adequate glycine to meet metabolic needs (including protein synthesis) of piglets. Based on these lines of compelling evidence, we conclude that glycine is a nutritionally essential amino acid for maximal growth and development of milk-fed young pigs. Our results also have important implications for improving the nutrition of human infants, particularly low birth weight or preterm infants.

Acknowledgments We thank students in our laboratories for assistance in this research. This project was supported, in part, by the National Basic Research Program of China (2013CB127302), the National Natural Science Foundation of China (31172217 and 31272450), China Postdoctoral Science Foundation (2012T50163), the Chinese Universities Scientific Fund (2013RC002), the Program for New Century Excellent Talents in University (NCET-12-0522), the Program for Beijing Municipal Excellent Talents, National Research Initiative Competitive Grants from the Animal Growth and Nutrient Utilization Program (2008-35206-18764 and 2014-67015-21770) of the USDA National Institute of Food and Agriculture, and Texas A&M AgriLife Research (H-8200).

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

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