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ORIGINAL ARTICLE

L-Glutamine or L-alanyl-L-glutamine prevents oxidant- or endotoxin-induced death of neonatal enterocytes

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Abstract This study tested the hypothesis that L-glutamine (Gln) or L-alanyl-L-glutamine (Ala-Gln) prevents oxidant- or endotoxin-induced death of neonatal enterocytes. Enterocytes of neonatal pigs rapidly hydrolyzed Ala-Gln and utilized Gln. To determine whether Gln or Ala-Gln has a cytoprotective effect, IPEC-1 cells were cultured for 24 h in Gln-free Dulbecco's modified Eagle's-F12 Ham medium containing 0, 0.5, 2.0 or 5.0 mM Gln or Ala-Gln, and 0, 0.5 mM H_2O_2 or 30 ng/ml lipopolysaccharide (LPS). Without Gln or Ala-Gln, H_2O_2- or LPS-treated cells exhibited almost complete death. Gln or Ala-Gln at 0.5, 2 and 5 mM dose-dependently reduced H_2O_2- or LPS-induced cell death by 14, 54 and 95%, respectively, whereas D-glutamine, alanine, glutamate, ornithine, proline, glucosamine or nucleosides had no effect. To evaluate the effectiveness of Gln or Ala-Gln in vivo, 7-day-old piglets received one-week oral administration of Gln or Ala-Gln (3.42 mmol/kg body weight) twice daily and then a single intraperitoneal injection of LPS (0.1 mg/kg body weight); piglets were euthanized in 24 and 48 h to analyze intestinal apoptotic proteins and morphology. Administration of Gln or Ala-Gln to LPS-challenged piglets increased Gln concentrations in small-intestinal lumen and plasma, reduced intestinal expression of Toll-like receptor-4, active caspase-3 and NF*k*B, ameliorated intestinal injury, decreased rectal temperature, and enhanced growth performance. These results demonstrate a protective effect of Gln or Ala-Gln against H_2O_2- or LPS-induced enterocyte death. The findings support addition of Gln or Ala-Gln to current Gln-free pediatric amino acid solutions to prevent intestinal oxidative injury and inflammatory disease in neonates.

Keywords Glutamine · Alanyl-glutamine · Endotoxin · Intestinal cells · Oxidative injury

Abbreviations

AA	Amino acid
Ala	L-Alanine
Ala-Gln	L-Alanyl-L-glutamine
DMEM-F12	Dulbecco's modified Eagle's F12 Ham medium
FBS	Fetal bovine serum
Gln	L-Glutamine
IPEC-1	Intestinal porcine epithelial cell line-1
LPS	Lipopolysaccharide
MyD88	Myeloid differentiation factor 88
NF*k*B	Nuclear factor kappa-light-chain enhancer of activator B cells
TLR4	Toll-like receptor 4

Unless indicated, amino acids used and analyzed in this study are L-isomers.

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Introduction

L-Glutamine (Gln) is a conditionally essential amino acid (AA) for infants and piglets (Reeds and Burrin 2001; Wu 1998). As a major fuel for absorptive epithelial cells of the small intestine (enterocytes), Gln plays an important role in maintaining gut integrity and preventing the entry of luminal pathogenic microorganisms into the systemic circulation (Blachier et al. 2007; Wang et al. 2008a). Gln is also required for the synthesis of nucleotides to support intestinal cell proliferation (Rhoads et al. 1997). In addition, Gln promotes intestinal restitution by activating mTOR signaling and expression of ornithine decarboxylase, a rate-controlling enzyme in the synthesis of polyamines (substances essential for cell proliferation and differentiation) (Wang 2007; Wu et al. 2007). Moreover, Gln may protect colonic epithelial cells and IEC-18 cells (a cell line derived from the rat ileum) from cytokine- and NH_2Cl-induced apoptosis, respectively (Evans et al. 2003; Wischmeyer et al. 1997). Owing to higher rates of Gln utilization by neonates compared with adults (Wu et al. 1995, 2008b), Gln requirements are likely greater in young mammals (Deng et al. 2008; Han et al. 2008; Wang et al. 2008a). However, current infant formulas contain no Gln because of concern over its degradation (albeit at a low rate) during a prolonged period of storage to form pyroglutamate (a potentially toxic substance for the brain).

Concentrations of free Gln in milk increase markedly with advanced lactation. For example, concentrations of free Gln in human milk increase from 24 μM within the first days of lactation to 560 μM at 3 months (Agostoni et al. 2000). Higher concentrations of free Gln (1.5–2 mM) have been reported for mature human milk (Neville and Neifert 1983). Similarly, we found that concentrations of free Gln in sow's milk increase from 0.1 mM on day 1 of lactation to 3.4 mM on day 29 of lactation (Wu and Knabe 1994). Gln is stable in the lumen of the neonatal stomach and small intestine (Wu et al. 1996), and thus free Gln in milk is fully available to enterocytes. Additionally, through the action of proteases and peptidases in the lumen of the gastrointestinal tract, milk protein is hydrolyzed to form tripeptides, dipeptides, and free AA for direct absorption into enterocytes (Wu et al. 2007). Because the digestibility of peptide-bound and free Gln in milk is approximately 100% in neonates (Lin et al. 2008), nearly all milk-derived Gln is absorbed by the neonatal small intestine.

We hypothesized that Gln or its chemically stable dipeptide L-alanyl-L-glutamine (Ala-Gln) may prevent oxidant or lipopolysaccharide (LPS)-induced death of neonatal enterocytes. This hypothesis was tested using cell cultures and the neonatal pig, which is an excellent animal model for studying infant nutrition (Ou et al. 2007; Suryawan et al. 2008).

Materials and methods

Materials

Fetal bovine serum (FBS) and antibiotics were procured from Invitrogen (Grand Island, NY, USA), whereas Gln and Ala-Gln were products of Ajinomoto Co., Inc. (Tokyo, Japan). HPLC-grade methanol and water were purchased from Fisher Scientific (Houston, TX, USA). Selenium and epidermal growth factor were obtained from BD Biosciences (Bedford, MA, USA), and plastic culture plates were manufactured by Corning Inc. (Corning, NY, USA). Unless indicated, all other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

Preparation of porcine jejunal enterocytes and culture of IPEC-1 cells

Viable jejunal enterocytes were isolated from 7-day-old sow-reared pigs using calcium-free Krebs buffer (Wu et al. 1994). Because enterocytes freshly isolated from piglets are biochemically viable for only up to 45 min (Wu et al. 1995), they are not suitable for evaluating efficacy of Gln or Ala-Gln in preventing oxidative or inflammatory stress. To overcome this problem, we used IPEC-1 cells derived from the jejunum of unsuckled newborn piglets using selective subculture techniques (Lu et al. 2002). IPEC-1 cells were generously provided by Dr. Dennis Black (University of Tennessee, Memphis, TN, USA). All cell cultures were carried out at 37°C in an atmosphere containing 5% CO_2. IPEC-1 cells were grown in serial passage in uncoated plastic culture flasks (100 cm^2) in DMEM-F12 containing 17.5 mM D-glucose, 2 mM Gln, 0.7 mM arginine, 15 mM HEPES (pH 7.4), 5% FBS, insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), epidermal growth factor (5 μg/L), penicillin (50 μg/ml), streptomycin (4 μg/ml), and 0.25 μg/ml amphotericin B (fungizone®). Medium was changed every 2 days. Cells were passaged using trypsinization. IPEC-1 cells from passages 30–50 were used in the studies.

Metabolism of Gln and Ala-Gln in intestinal cells

Freshly isolated enterocytes and IPEC-1 cells (~5 mg cell protein) were incubated at 37°C for 45 min in 2-ml oxygenated (95% O_2/5% CO_2) Krebs buffer containing 1% bovine serum albumin, 20 mM HEPES (pH 7.4), 5 mM D-glucose, 0, 0.5, 2, or 5 mM Gln, alanine (Ala) or Ala-Gln. The concentrations of Gln and Ala were chosen on the basis of those in the jejunum lumen of milk-fed piglets (Wu et al. 1995). The incubation was terminated by addition of 200 μl of 1.5 M $HClO_4$, and the neutralized extracts were analyzed for AA and Ala-Gln using HPLC (Li et al. 2008a;

Wu and Meininger 2008). Ammonia in the incubation medium was determined using glutamate dehydrogenase (Wu et al. 1995). Disappearance of Gln and Ala-Gln from the incubation medium was taken to indicate their utilization. Rates of product formation from Gln or Ala-Gln were calculated on the basis of differences in concentrations of metabolites in incubation medium plus cell extracts between the presence and absence of the added substrate.

Effects of Gln and Ala-Gln on H_2O_2- or LPS-induced death of neonatal enterocytes

To determine effects of Gln or Ala-Gln on H_2O_2-induced cell death, confluent IPEC-1 cells were cultured for 24 h at 37°C in Gln-free DMEM-F12 containing 17.5 mM D-glucose, 0.7 mM arginine, 15 mM HEPES (pH 7.4), 5% FBS, insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), epidermal growth factor (5 μg/L), penicillin (50 μg/ml), streptomycin (4 μg/ml), 0.25 μg/ml amphotericin B (fungizone®), 0, 0.5, 2.0, or 5.0 mM Gln or Ala-Gln, and 0, 0.5 mM H_2O_2 or 30 ng/ml LPS. In additional experiments, 2 μM L-buthionine sulfoximine (a specific inhibitor of γ-glutamyl cysteine ligase, which is a key regulatory enzyme in glutathione synthesis; Evans et al. 2003) was added to culture medium containing 2 mM Gln. To determine whether Gln metabolism was necessary for Gln's effect on cell survival, the Gln- or Ala-Gln-free culture medium contained: (1) 0.5 or 2 mM D-glutamine, glutamate, Ala, ornithine, aspartate, or glucosamine; (2) a mixture of nucleosides plus bases (adenosine, guanosine, cytosine, inosine, and uridine) (0.1 or 0.5 mM each); or (3) 0.1 or 1 mM azaserine or 6-diazo-5-oxo-L-norleucine (inhibitors of glutamine:fructose-6-phosphate transaminase and phosphate-activated glutaminase). After 24-h culture, cells were gently washed with Dulbecco's phosphate buffered saline containing 2.5 mM calcium. Three random areas of each plate were photographed for counting viable cells using a Nikon FA camera (Nikon Inc., Melville, NY, USA) attached to an Axiovert 100 (Zeiss) microscope (Carl Zeiss MicroImaging GmbH, Munich, Germany) with a 16× objective.

Concentrations of Gln in proteins plus peptides of porcine milk

To determine an optimal dose of supplemental Gln for sow-reared piglets, it is important to determine concentrations of protein- and peptide-bound Gln and glutamate in porcine milk. Because Gln and glutamate are closely related to asparagine and aspartate in cell metabolism (Wu et al. 2007), it is also necessary to analyze their concentrations in milk protein. Thus, we quantified Gln, glutamate, asparagine, and aspartate in porcine milk proteins plus peptides using the enzymatic hydrolysis method of Tsao and Otter (1999), with modifications. All procedures were performed under strict sterile conditions to prevent bacterial contamination.

Colostrum (6–10 h after farrowing) and milk (days 3–28 of lactation) were obtained from lactating sows fed a corn- and soybean-based diet (Wang et al. 2008b), as described by Wu and Knabe (1994). The day of farrowing was taken as day 1 of lactation. An aliquot (25 μl) of a whole-milk sample or 25 μl of H_2O (blank) in glass tubes was placed in a 100°C water-bath for 5 min to kill bacteria. The tubes were cooled to room temperature, followed by addition of 100 μl of 0.1 mM Hepes buffer (pH 7.5) plus 0.275% sodium azide plus antibiotics (50 μg/ml penicillin, 4 μg/ml streptomycin, and 0.25 μg/ml amphotericin B), 50 μl of 2 mg/ml Pronase E (from *Streptomyces griseus*; Sigma Cat #P5147), 25 μl of 2 mg/ml prolidase (from porcine kidney; Sigma Cat #P6675), 25 μl of 2 mg/ml pyroglutamate aminopeptidase (from *Bacillus amyloliquefaciens*; Sigma Cat #P4669), 25 μl of 2 mg/ml carboxypeptidase A (from bovine pancreas; Sigma Cat #C0261), and 25 μl of 2 mg/ml aminopeptidase M (from porcine kidney; Sigma Cat #L0632). After the tubes were incubated at 37°C for 24 h, the reaction was terminated by addition of 50 μl of 1.5 M $HClO_4$. Neutralized extracts were analyzed for AA (Wu and Meininger 2008). Using this enzymatic hydrolysis method, we found that the recovery of Gln from bovine α-lactalbumin, β-lactoglobulin, and β-casein standards was 91.5, 98.3, and 99.6%, respectively; the recovery of asparagine from bovine α-lactalbumin, β-lactoglobulin, and β-casein standards was 93.1, 91.4, and 99.5%, respectively; the recovery of glutamate from bovine α-lactalbumin, β-lactoglobulin, and β-casein standards was 52.8, 67.2, and 51.4%, respectively; and the recovery of aspartate from bovine α-lactalbumin, β-lactoglobulin, and β-casein standards was 50.4, 71.0, and 78.6%, respectively. Acid hydrolysis of milk under standard conditions (6 M HCl at 110°C for 24 h) was performed to obtain total glutamate plus Gln and total aspartate plus asparagine in proteins plus peptides, whereas free Gln, glutamate, asparagine, and aspartate in porcine milk were determined by HPLC (Wu and Knabe 1994). The amounts of peptide-bound Gln were calculated by subtracting free Gln from total Gln in milk. Due to the relatively low recovery of peptide-bound glutamate, the amounts of peptide-bound glutamate were calculated by subtracting free plus peptide-bound Gln and free glutamate from total glutamate plus Gln. Similar calculations were applied to the analysis of asparagine and aspartate in proteins plus peptides.

Effects of Gln or Ala-Gln on LPS-induced intestinal injury in piglets

Sows were fed daily a 2-kg gestating diet during the entire period of pregnancy that met the National Research

Council (NRC)-recommended requirements of nutrients (Self et al. 2004). After farrowing, sows had free access to a corn- and soybean meal-based diet that also met NRC-recommended requirements of nutrients (Wang et al. 2008b). Each sow nursed nine piglets. All sows had free access to drinking water. Neonatal pigs were used for three series of experiments.

In Exp 1, piglets with similar body weight from 10 litters (5 piglets/litter) were assigned randomly to five groups on the basis of litter origin and sex ($n = 10$/group; 5 males and 5 females). Beginning at 7 days of age, piglets in groups 1, 2, 3, 4, and 5 received oral administration of water, water, Ala, Gln, and Ala-Gln (3.42 mmol/kg body weight), respectively, twice daily (8:00 a m. and 5:00 p.m.). AA was dissolved in water, and the volume of administered solution was 20 ml per piglet. The dose of supplemental Gln was equivalent to 73% of daily Gln intake from sow's milk by the 7-day-old piglet weighing 2.5 kg. In our preliminary study, we found that oral administration of 6.84 mmol or 1 g Gln/kg body weight to 7- to 21-day-old sow-reared piglets twice daily reduced ($P < 0.01$) daily weight gain by 19%, compared with piglets receiving an isonitrogenous amount of Ala (225.0 ± 3.9 vs 181.4 ± 3.4 g/day; $n = 20$); therefore, a lower dose of Gln was used in the present study. At 14 days of age, piglets in groups 2–5 received a single intraperitoneal injection of LPS (0.1 mg/kg body weight), whereas piglets in group 1 received a single intraperitoneal injection of saline. The dose of LPS was based on the study of Orellana et al. (2008). At 0, 2, 6, 12, 24, and 48 h post LPS injection, rectal temperatures were determined using a thermometer. All piglets had free access to sows. At 16 days of age, 1 h after oral administration of Gln or Ala-Gln, blood samples (~3 ml) were obtained from the jugular vein of each piglet into a heparinized tube containing potassium EDTA (2 mg/ml). Piglets were then euthanized to obtain the intestine, as we described (Wu et al. 1995).

For glutathione assays, 0.2 ml of whole blood was mixed with 0.2 ml of solution A (1 mg sodium heparin, 10 mg serine and 4.5 mg iodoacetic acid in 2 ml of 100 mM sodium borate). The tubes were centrifuged at 10,000*g* for 1 min and 0.2 ml of the supernatant fluid was mixed with 0.1 ml of solution B (1.5 M $HClO_4$ and 0.2 M boric acid), followed by addition of 50 µl of 2 M K_2CO_3. Neutralized extracts were analyzed for reduced glutathione and oxidized glutathione (Wang et al. 2008b). For analysis of adenyl purines, 0.2 ml of plasma was mixed with 0.2 ml of 1.5 M $HClO_4$ and then 0.1 ml of 2 M K_2CO_3; extracts were obtained after centrifugation at 12,000*g* for 1 min and stored at −80°C until analysis.

The whole small intestine was weighed and its length was measured after careful removal of luminal contents. The luminal contents were centrifuged at 12,000*g* for 1 min, and the supernatant fluid was placed in liquid nitrogen and stored at –80°C. A portion of mid-jejunum (~3 cm each in length) was placed in 4% paraformaldehyde for subsequent analysis of morphology (Wu et al. 1996), and sets of samples (~10 cm each in length) were obtained for glutathione analysis (Wang et al. 2008b). For analysis of adenyl purines, a frozen tissue (~0.1 g) was homogenized in 1 ml of 1.5 M $HClO_4$ using a glass homogenizer. The homogenate was transferred to a 15-ml tube. The homogenizer was rinsed with 1 ml of 1.5 M $HClO_4$, followed by addition of 0.5 ml of 2 M K_2CO_3. All the tubes were centrifuged at 3,000*g* for 5 min, and the supernatant fluid was used for determination of ATP, ADP, AMP, adenine, adenosine, and cAMP.

Exp 2 was conducted as Exp 1, except that milk consumption by suckling piglets was measured over an 8-h period at day 10 (3 days after initiation of Gln administration) and day 15 (24 h after LPS injection), using the weigh-suckle-weigh technique (Kim and Wu 2008; Wu et al. 2000). Exp 3 was conducted as Exp 1, except that piglets were euthanized at 24 h after LPS injection to obtain the jejunum for determination of apoptotic proteins.

Analysis of adenyl purines

Adenyl purines were determined using the method of Katayama et al. (2001), with modifications. An aliquot (100 µl) of sample or standard solution (1 µM each of ATP, ADP, AMP, adenine, adenosine, and cAMP) was mixed with 10 µl of 4 M 2-chloroacetaldehyde and 100 µl of 1 M acetate buffer (pH 4.5). All the tubes were placed in an 80°C water-bath for 20 min, followed by centrifugation at 10,000*g* for 2 min. The supernatant fluid was transferred to a micro-insert tube (placed in a brown vial) for analysis using a Waters HPLC system (Wu and Meininger 2008), an analytical column (Capcell Pak C18 SG120; 150 × 4.6 mm, 5 µm; Shiseido, Tokyo, Japan) guarded by a Supelco C18 column (50 × 4.6 mm, 40 µm; Bellefonte, PA, USA), and a Model 2475 Multi λ Fluorescence Detector (280-nm excitation and 420-nm emission; gain setting at 1,000). The volume of sample injected into the HPLC column was 10 µl and total running time (including column regeneration) was 60 min. The mobile-phase gradient was as follows: 0–7 min, 100% A (50 mM citric acid, 100 mM disodium hydrogen phosphate, and 3% methanol; pH 4.5), 0.4 ml/min; 7.1–11 min, 96% A and 4% B (100% methanol), 0.55 ml/min; 11.1–44 min, 96% A and 4% B, 1.0 ml/min; 44.1–48 min, 100% B, 1.0 ml/min; 48.1–60 min, 100% A, 1.2 ml/min. The retention times of ATP, ADP, AMP, adenine, adenosine, and cAMP were 9.5, 11.4, 23.4, 28.6, 33.9, and 42.1 min, respectively. Adenyl purines in samples were quantified on the basis of authentic standards using the

Millenium-32 workstation (Waters Inc., Milford, MA, USA). The recovery of adenyl purines from plasma and intestinal tissue homogenates, as measured with authentic standards, was 93–97 and 91–94%, respectively.

Western blot analysis of apoptotic proteins

Jejunal tissue was pulverized in liquid nitrogen and homogenized in lysis buffer containing 20 mM Tris–HCl (pH 7.4), 50 mM NaCl, 50 mM NaF, 50 mM EDTA, 1% Triton X-100, 1× protease inhibitor cocktail, and 1× phosphatase inhibitor cocktail (Calbiochem, La Jolla, CA). Proteins in homogenates were determined using the Pierce BCA Protein Assay Kit. Western blot analyses of TLR4, active caspase-3, and NF*k*B proteins were performed (Li et al. 2008b), using anti-TLR4 (Cat #ab53629, Abcam, Cambridge, MA, USA), anti-active caspase-3 (17 kDa; Cat #9661, Cell Signaling, Beverly, MA, USA), and anti-NFkB p65 (Cat #3034, Cell Signaling), respectively. Results were expressed as relative intensities (arbitrary unit) and were normalized to the values for saline-injected piglets receiving oral water administration.

Statistical analysis

Values are expressed as mean ± SEM. Data were analyzed as a randomized block design by analysis of variance, with sow as the block. Differences among treatment means were determined by the Tukey multiple comparison test. All statistical analyses were performed using SAS (SAS Institute Inc., Cary, NC, USA). P values ≤ 0.05 were taken to indicate statistical significance.

Results

Utilization of Gln and Ala-Gln by intestinal cells

Very small amounts of Ala and glutamate were released from jejunal enterocytes into incubation medium in the absence of extracellular Gln or Ala-Gln (Table 1). However, these cells rapidly hydrolyzed Ala-Gln and subsequently converted Gln into glutamate (Table 1). Approximately 60, 93 and 96% of the dipeptide was hydrolyzed to form Ala plus Gln within 5, 15 and 30 min, respectively. Thus, most of the Ala-Gln disappeared from

Table 1 Concentrations of Ala-Gln and AA in the medium of porcine jejunal enterocytes incubated for 5–45 min with 0 or 5 mM Ala-Gln

Time (min)	0 mM Ala-Gln		5 mM Ala-Gln				
	Alanine	Glutamate	Ala-Gln	Alanine	Glutamine	Glutamate	Gln + Glu
5	3.48 ± 0.41^{d}	3.31 ± 0.39^{d}	2028 ± 84^{a}	2986 ± 78^{b}	2874 ± 71^{c}	77 ± 3.4^{d}	2951 ± 70^{b}
15	11.6 ± 0.83^{c}	9.89 ± 0.72^{c}	341 ± 12^{b}	4669 ± 90^{a}	4393 ± 84^{a}	225 ± 5.1^{c}	4618 ± 86^{a}
30	19.2 ± 1.36^{b}	19.2 ± 1.16^{b}	202 ± 7.3^{c}	4805 ± 81^{a}	4351 ± 95^{a}	434 ± 12^{b}	4785 ± 91^{a}
45	31.4 ± 2.88^{a}	27.0 ± 1.48^{a}	188 ± 7.0^{c}	4819 ± 93^{a}	4062 ± 88^{b}	683 ± 17^{a}	4745 ± 93^{a}

Values (μM) are mean ± SEM, $n = 8$. Jejunal enterocytes were isolated from 7-day-old pigs and incubated at 37°C for 5–45 min in 2 ml of Krebs bicarbonate buffer containing approximately 5 mg cell protein and 0 or 5 mM Ala-Gln. *a–d* Mean sharing different superscript letters within a column differ ($P < 0.01$). Concentration of Gln in the incubation medium without Ala-Gln was not detected (< 0.1 μM)

Table 2 Metabolism of Gln and Ala-Gln in jejunal enterocytes of 7-day-old piglets

Medium Gln or Ala-Gln	Utilization of Gln or hydrolysis of Ala-Gln	Production of metabolites							
		Ammonia	Glu	Ala	Asp	Orn	Cit	Arg	Pro
0.5 mM Gln	68.4 ± 5.3^{c}	71.2 ± 4.5^{c}	30.9 ± 2.1^{c}	4.06 ± 0.2^{c}	6.52 ± 0.5^{c}	0.10 ± 0.01^{c}	0.58 ± 0.06^{c}	0.36 ± 0.04^{c}	1.01 ± 0.12^{c}
2 mM Gln	223 ± 13.7^{b}	236 ± 16^{b}	102 ± 7.7^{b}	30.3 ± 2.0^{b}	21.7 ± 1.2^{b}	0.28 ± 0.03^{b}	1.94 ± 0.11^{b}	1.21 ± 0.13^{b}	3.48 ± 0.32^{b}
5 mM Gln	493 ± 21.5^{a}	511 ± 22^{a}	213 ± 17^{a}	64.5 ± 4.1^{a}	34.8 ± 2.7^{a}	0.61 ± 0.05^{a}	4.47 ± 0.34^{a}	2.62 ± 0.28^{a}	7.63 ± 0.66^{a}
0.5 mM Ala-Gln	196 ± 10^{c}	68.6 ± 4.0^{c}	31.4 ± 2.5^{c}	193 ± 8.5^{c}	6.71 ± 0.6^{c}	0.11 ± 0.01^{c}	0.54 ± 0.05^{c}	0.33 ± 0.03^{c}	1.12 ± 0.14^{c}
2 mM Ala-Gln	825 ± 37^{b}	219 ± 14^{b}	103 ± 7.8^{b}	830 ± 41^{b}	22.4 ± 1.7^{b}	0.30 ± 0.02^{b}	1.80 ± 0.13^{b}	1.15 ± 0.10^{b}	3.56 ± 0.29^{b}
5 mM Ala-Gln	2140 ± 86^{a}	496 ± 25^{a}	208 ± 14^{a}	2134 ± 66^{a}	33.9 ± 2.5^{a}	0.64 ± 0.04^{a}	4.29 ± 0.38^{a}	2.49 ± 0.22^{a}	7.81 ± 0.83^{a}

Values, expressed as nmol/(mg protein x 45 min), are means ± SEM, n = 8. Jejunal enterocytes were isolated from 7-day-old pigs and incubated at 37°C for 45 min in 2 ml of Krebs bicarbonate buffer containing ~ 5 mg cell protein and 0 to 5 mM Gln, or 0 to 5 mM Ala-Gln. Mean sharing different superscript letters within a column for the Gln or Ala-Gln treatment differ ($P < 0.01$)

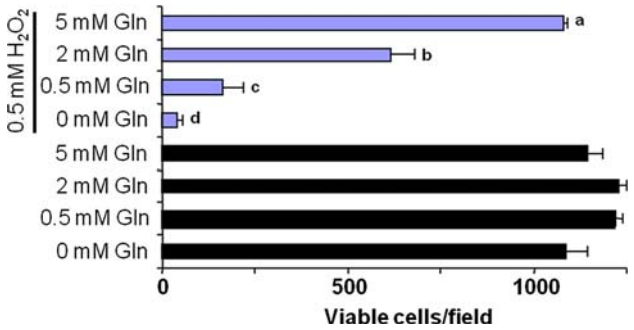

Fig. 1 Addition of 0.5, 2 or 5 mM Gln to Gln-free medium dose-dependently reduced the death of IPEC-1 cells cultured for 24 h in the presence of 0.5 mM H_2O_2. *a–d* Mean with different letters differ ($P < 0.01$)

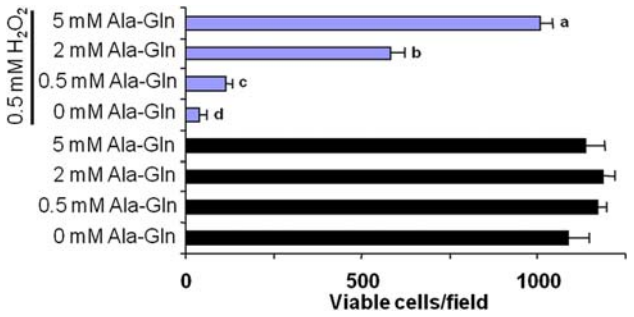

Fig. 2 Addition of 0.5, 2 or 5 mM Ala-Gln to Gln-free medium dose-dependently reduced the death of IPEC-1 cells cultured for 24 h in the presence of 0.5 mM H_2O_2. *a–d* Mean with different letters differ ($P < 0.01$)

incubation medium within 5 min of exposure to jejunal enterocytes, with the concomitant increase in Ala, Gln and glutamate in the medium. At each time point, the rate of Ala-Gln hydrolysis did not differ from the rate of Ala accumulation in jejunal enterocytes. Similar results were obtained for IPEC-1 cells (data not shown).

Extracellular Gln or dipeptide-derived Gln was extensively catabolized by jejunal enterocytes to produce ammonia, glutamate, Ala, aspartate, ornithine, citrulline, arginine, and proline (Table 2). Increasing extracellular concentrations of Gln or Ala-Gln from 0.5 to 5 mM increased ($P < 0.01$) the rates of Gln degradation in a dose-dependent manner. There was detectable production of asparagine by jejunal enterocytes incubated in the presence of 5 mM Gln or Ala-Gln (0.69 ± 0.06 and 0.66 ± 0.08 nmol/mg protein per 45 min, respectively), but not in the presence of 0.5 or 2 mM Gln or Ala-Gln. However, there was no detectable formation of ammonia, aspartate, glutamate, asparagine, citrulline, ornithine, arginine or proline (<0.1 nmol/mg protein per 45 min) from 0.5, 2 or 5 mM Ala in jejunal enterocytes. Similar results were obtained for IPEC-1 cells (data not shown).

Effects of Gln and Ala-Gln on H_2O_2- or LPS-induced enterocyte death

In the absence of H_2O_2, the number of viable IPEC-1 cells did not differ among the 0, 0.5, 2 and 5 mM Gln groups

Table 3 Concentrations of free and protein-bound Gln, glutamate, asparagine, and aspartate in sow's colostrum and milk

Amino acid	Days of lactation					
	1	3	8	15	22	28
Free glutamine	0.015 ± 0.003^{e}	0.052 ± 0.010^{d}	0.181 ± 0.029^{c}	0.188 ± 0.017^{c}	0.303 ± 0.041^{b}	0.532 ± 0.046^{a}
Free glutamate	0.022 ± 0.005^{c}	0.061 ± 0.008^{b}	0.167 ± 0.014^{a}	0.173 ± 0.019^{a}	0.158 ± 0.012^{a}	0.161 ± 0.014^{a}
Protein-bound Gln[1]	10.8 ± 1.24^{a}	6.97 ± 0.76^{b}	4.22 ± 0.51^{c}	4.10 ± 0.45^{c}	4.03 ± 0.39^{c}	3.75 ± 0.30^{c}
Protein-bound Glu[2]	13.2 ± 1.56^{a}	7.93 ± 0.82^{b}	4.56 ± 0.39^{c}	4.58 ± 0.53^{c}	4.37 ± 0.34^{c}	4.23 ± 0.32^{c}
Total Gln	10.8 ± 1.24^{a}	7.02 ± 0.77^{b}	4.40 ± 0.48^{c}	4.29 ± 0.47^{c}	4.33 ± 0.37^{c}	4.28 ± 0.35^{c}
Total Glu	13.2 ± 1.56^{a}	7.99 ± 0.81^{b}	4.72 ± 0.41^{c}	4.75 ± 0.56^{c}	4.53 ± 0.32^{c}	4.39 ± 0.34^{c}
Total Gln + Glu	24.0 ± 2.2^{a}	15.0 ± 1.8^{b}	9.13 ± 1.0^{c}	9.04 ± 0.85^{c}	8.86 ± 0.50^{c}	8.67 ± 0.62^{c}
Free asparagine	<0.001	0.004 ± 0.001^{c}	0.015 ± 0.003^{b}	0.017 ± 0.002^{b}	0.028 ± 0.004^{b}	0.031 ± 0.004^{a}
Free aspartate	0.005 ± 0.001^{c}	0.026 ± 0.003^{b}	0.068 ± 0.007^{a}	0.063 ± 0.007^{a}	0.066 ± 0.008^{a}	0.065 ± 0.005^{a}
Protein-bound Asn[3]	7.46 ± 0.91^{a}	4.55 ± 0.68^{b}	2.41 ± 0.27^{c}	2.36 ± 0.23^{c}	2.33 ± 0.25^{c}	2.12 ± 0.22^{c}
Protein-bound Asp[4]	8.04 ± 1.27^{a}	4.26 ± 0.49^{b}	2.47 ± 0.29^{c}	2.24 ± 0.20^{c}	2.20 ± 0.22^{c}	2.28 ± 0.24^{c}
Total Asn	7.46 ± 0.91^{a}	4.55 ± 0.68^{b}	2.43 ± 0.26^{c}	2.38 ± 0.24^{c}	2.36 ± 0.24^{c}	2.15 ± 0.23^{c}
Total Asp	8.05 ± 1.27^{a}	4.29 ± 0.48^{b}	2.54 ± 0.30^{c}	2.30 ± 0.21^{c}	2.27 ± 0.21^{c}	2.35 ± 0.25^{c}
Total Asn + Asp	15.5 ± 1.83^{a}	8.84 ± 0.91^{b}	4.96 ± 0.66^{c}	4.68 ± 0.27^{c}	4.62 ± 0.40^{c}	4.50 ± 0.36^{c}

Values expressed as g/L of whole milk, are mean ± SEM, $n = 10$. Mean sharing different superscript letters within a row differ ($P < 0.01$)

[1] Glutamine in proteins and peptides of milk

[2] Glutamate in proteins and peptides of milk

[3] Asparagine in proteins and peptides of milk

[4] Aspartate in proteins and peptides of milk

(Fig. 1) or the 0, 0.5, 2 and 5 mM Ala-Gln groups (Fig. 2). IPEC-1 cells exposed to 0.5 mM H_2O_2 without Gln or Ala-Gln exhibited 3.5% survival rate. Importantly, addition of 0.5, 2, and 5 mM Gln to culture medium reduced ($P < 0.01$) H_2O_2-induced cell death by 14.2, 54.4, and 95.4%, respectively, compared with control cells without H_2O_2 (Fig. 1). Likewise, supplementing 0.5, 2, and 5 mM Ala-Gln to the culture medium reduced ($P < 0.01$) H_2O_2-induced cell death by 13.5, 53.0, and 94.2%, respectively, in comparison with control cells without H_2O_2 (Fig. 2). The number of viable cells did not differ between 5 mM Gln and 5 mM Gln + 0.5 mM H_2O_2 groups or between 5 mM Ala-Gln and 5 mM Ala-Gln + 0.5 mM H_2O_2 groups. Gln was still effective in preventing H_2O_2-induced cell death by 95 ± 3.8% even in the presence of 2 μM L-buthionine sulfoximine that reduced the concentration of reduced glutathione in cells by 32 ± 2.4%. Interestingly, H_2O_2-induced enterocyte death could not be ameliorated by addition to the Gln- or Ala-Gln-free culture medium containing (1) 0.5 or 2 mM D-glutamine, glutamate, Ala, ornithine, aspartate, glucosamine; (2) a mixture of nucleosides plus bases (adenosine, guanosine, cytosine, inosine, and uridine) at 0.1 or 0.5 mM each; and (3) 0.1 or 1 mM azaserine or 6-diazo-5-oxo-L-norleucine (data not shown). Similar results were obtained for LPS-treated cells (data not shown).

Concentrations of protein- and peptide-bound Gln in sow's milk

The ratios of Gln to glutamate in proteins plus peptides of porcine colostrum and milk were 0.818 and 0.925, respectively (Table 3). Concentrations of protein- and peptide-bound Gln and glutamate decreased ($P < 0.01$) progressively between days 1 and 8 of lactation and did not differ between days 8 and 29 of lactation. Free Gln was only 0.14% of protein- and peptide-bound Gln on day 1 of lactation, but increased ($P < 0.01$) progressively with advanced lactation stage. On day 29 of lactation, free Gln accounted for 14% of protein- and peptide-bound Gln. Sow's mature milk (on days 8–29 of lactation) contained 4.33 g total Gln, 4.60 g total glutamate, 2.33 g total asparagine, and 2.37 g total aspartate per L of whole milk (Table 3). Concentrations of total Gln and total asparagine (free plus protein- and peptide-bound) in colostrum were 2.5 and 3.2 times those in mature milk, respectively.

Effects of oral Gln or Ala-Gln administration on piglets before and after LPS challenge

Before LPS injection, oral administration of Gln or Ala-Gln for 1 week did not affect the milk intake of piglets but increased ($P < 0.01$) their growth performance by 12%,

Table 4 Growth, rectal temperature, small intestine weight, and jejunal morphology of LPS-challenged piglets receiving oral administration of water, Ala, Gln or Ala-Gln

Treatment	BW at day 7 of age	Milk intake of piglets		Change in BW between days		Change in RT	Small intestine at day 16		Jejunal morphology at day 16		
		Day 10† (ml/kg BW/8 h)	Day 15‡ (ml/kg BW/8 h)	7–14† (g/day)	14–16¶ (g/day)	0–12 h§ (°C)	Weight (g)	Length (cm)	VH (μm)	CD (μm)	LPD
	(kg)										
H_2O + saline	2.58 ± 0.10	96 ± 5.7	92^a ± 5.2	212^b ± 3.3	234^a ± 3.6	0.10^c ± 0.02	130^a ± 2.4	663 ± 29	542^a ± 23	190 ± 8.4	229 ± 9.2
H_2O + LPS	2.56 ± 0.09	90 ± 5.2	69^b ± 4.8	206^b ± 3.5	20^c ± 1.2	1.54^a ± 0.07	103^b ± 1.9	651 ± 26	413^b ± 18	178 ± 9.1	207 ± 12
Ala + LPS	2.59 ± 0.14	98 ± 6.6	66^b ± 5.1	210^b ± 3.2	18^c ± 1.4	1.57^a ± 0.08	96^b ± 2.2	658 ± 25	419^b ± 21	174 ± 8.8	210 ± 9.5
Gln + LPS	2.56 ± 0.11	93 ± 5.4	75^b ± 6.5	237^a ± 2.8	86^b ± 4.5	1.02^b ± 0.06	125^a ± 2.5	668 ± 33	527^a ± 24	180 ± 8.6	214 ± 11
Ala-Gln + LPS	2.60 ± 0.12	92 ± 6.0	72^b ± 6.2	235^a ± 3.0	82^b ± 3.7	0.96^b ± 0.03	131^a ± 2.7	660 ± 27	531^a ± 19	182 ± 9.7	218 ± 13

Values are mean ± SEM, $n = 10$ for all parameters except for body weights ($n = 20$). Mean sharing different superscript letters within a column differ ($P < 0.01$)

BW body weightl, *CD* crypt depth, *LPD* lamina propria depth, *RT* rectal temperature

† Measured before intraperitoneal injection of saline or LPS

‡ Measured at 24 h after intraperitoneal injection of saline or LPS

¶ Measured at 0 and 48 h after intraperitoneal injection of saline or LPS

§ Measured at 0 and 12 h after intraperitoneal injection of saline or LPS. Rectal temperature (38.9 ± 0.07°C) did not differ among the five groups of piglets before LPS injection

Table 5 Concentrations of free Gln and Ala in the plasma and jejunal lumen fluid of LPS-challenged piglets receiving oral administration of water, Ala, Gln or Ala-Gln

Treatment	Plasma (μM)		Jejunal lumen fluid (mM)	
	Gln	Ala	Gln	Ala
H_2O + saline	507 ± 16^c	490 ± 18^c	2.56 ± 0.23^c	2.84 ± 0.16^d
H_2O + LPS	403 ± 19^d	366 ± 15^d	2.33 ± 0.21^c	2.91 ± 0.18^d
Ala + LPS	426 ± 23^d	889 ± 41^b	2.48 ± 0.26^c	5.54 ± 0.39^b
Gln + LPS	874 ± 35^b	472 ± 28^c	4.52 ± 0.32^b	3.86 ± 0.25^c
Ala-Gln + LPS	1192 ± 57^a	2185 ± 92^a	5.78 ± 0.46^a	8.97 ± 0.62^a

Values are mean ± SEM, $n = 10$. Mean sharing different superscript letters within a column differ ($P < 0.01$). The jejunum and blood samples were obtained at 48 h after LPS injection (1 h after suckling and oral administration of water, Ala, Gln, or Ala-Gln)

compared with piglets receiving oral administration of water or Ala (Table 4). Oral Ala-Gln was more effective than oral Gln in increasing concentrations of free Gln in plasma and jejunal lumen (Table 5). Similarly, oral Ala-Gln enhanced concentrations of free Ala in plasma and jejunal lumen fluid to a greater extent than oral Ala.

Rectal temperature increased ($P < 0.01$) progressively in all groups of LPS-challenged piglets to the highest value at 12 h post injection, but did not change in saline-injected piglets during this period (Table 4). Oral administration of Gln or Ala-Gln reduced ($P < 0.01$) the LPS-induced increase in rectal temperature by 0.5°C. Rectal temperatures did not differ among all groups of piglets at 48 h post LPS injection.

There was little consumption of milk by all LPS-treated piglets within 12 h of the endotoxin challenge. However, at 24 h post LPS injection, piglets resumed milk consumption, although the amount was 20–25% lower ($P < 0.01$) than that of saline-injected piglets (Table 4). Growth performance was very poor in LPS-treated piglets receiving oral water or Ala administration, but markedly increased ($P < 0.01$) in response to Gln or Ala-Gln supplementation. Importantly, LPS-induced jejunal injury, cell death, and oxidative stress were completely prevented by oral Gln or Ala-Gln, as indicated by small intestine weights and jejunal villus height (Table 4), as well as the ratios of oxidized glutathione to reduced glutathione in plasma and jejunal tissue (Table 6). Plasma concentrations of all measured adenyl purines as well as jejunal tissue concentrations of ATP, AMP, adenosine and cAMP were increased ($P < 0.05$) in response to LPS injection, and these alterations were normalized by oral Gln or Ala-Gln (Table 6).

Expression of apoptotic proteins

Protein levels of Toll-like receptor-4, caspase-3, and NF*k*B were markedly increased ($P < 0.01$) in the jejunum of LPS-challenged piglets, compared with saline-injected controls (Table 7). Oral administration of Gln or Ala-Gln greatly reduced ($P < 0.01$) intestinal expression of these three proteins in LPS-treated piglets, but oral Ala had no effect (Table 7).

Discussion

Gln has versatile roles in nutrition and metabolism (Deldicque et al. 2008; Garcia et al. 2007, Lagranha et al. 2008b). The published work also shows that dietary supplementation with Gln effectively ameliorates intestinal atrophy, enhances the absorption of nutrients, and improves growth performance in early-weaned piglets (Wang et al. 2008b; Wu et al. 1996). Major findings of this study are that (1) addition of Gln or Ala-Gln to culture medium prevented the death of H_2O_2- or LPS-treated neonatal enterocytes and this effect was specific to the L-isomer; and (2) oral administration of Gln or Ala-Gln improved antioxidative capacity in enterocytes, reduced intestinal expression of apoptotic proteins, prevented intestinal damage, and ameliorated growth retardation in LPS-challenged piglets. Results from these in vitro and in vivo studies strongly support the use of Gln or Ala-Gln in the clinical management of endotoxin-infected infants and the rearing of neonatal pigs. To our knowledge, this is the first report regarding a beneficial effect of oral Gln or Ala-Gln administration on milk-fed neonates.

Gln is an abundant free AA in porcine milk (Wu and Knabe 1994). Enzymatic hydrolysis of peptide-bound Gln revealed that milk protein contained a large amount of Gln (Table 3), with the content of peptide-bound glutamate and Gln being similar throughout lactation (Table 3). Interestingly, the proportion of free Gln relative to total Gln in milk increased markedly between days 1 and 28 of lactation. Because the uptake of arterial Gln by the porcine mammary gland does not differ substantially between days 11 and 21 of lactation (Trottier et al. 1997), it is likely that rates of de novo synthesis of Gln in mammary tissue increase progressively with advanced lactation. The

Table 6 Concentrations of adenyl purines and ratios of oxidized glutathione to reduced glutathione in the plasma and jejunal tissue of LPS-challenged piglets receiving oral administration of water, Ala, Gln or Ala-Gln

Treatment	Plasma (μM)					Plasma GSHR	Jejunal tissue (nmol/g wet tissue)					Jejunal tissue
	ATP	ADP	AMP	Aden	cAMP		ATP	ADP	AMP	Aden	cAMP	GSHR
H_2O + saline	$7.52^{b} \pm 0.24$	$7.18^{b} \pm 0.28$	$1.44^{b} \pm 0.05$	$0.23^{b} \pm 0.01$	$0.28^{b} \pm 0.01$	$0.63^{b} \pm 0.03$	$2273^{a} \pm 87$	889 ± 43	$310^{b} \pm 12$	$140^{b} \pm 6.3$	$2.16^{b} \pm 0.13$	$0.054^{b} \pm 0.01$
H_2O + LPS	$9.10^{a} \pm 0.30$	$8.55^{a} \pm 0.32$	$1.83^{a} \pm 0.07$	$0.28^{a} \pm 0.02$	$0.35^{a} \pm 0.03$	$0.96^{a} \pm 0.05$	$1729^{b} \pm 72$	1020 ± 48	$374^{a} \pm 18$	$183^{a} \pm 7.1$	$2.88^{a} \pm 0.21$	$0.087^{a} \pm 0.02$
Ala + LPS	$8.95^{a} \pm 0.27$	$8.42^{a} \pm 0.34$	$1.95^{a} \pm 0.06$	$0.30^{a} \pm 0.01$	$0.37^{a} \pm 0.03$	$0.95^{a} \pm 0.04$	$1766^{b} \pm 68$	981 ± 55	$368^{a} \pm 17$	$189^{a} \pm 7.5$	$2.75^{a} \pm 0.17$	$0.089^{a} \pm 0.02$
Gln + LPS	$7.82^{b} \pm 0.25$	$7.39^{b} \pm 0.31$	$1.50^{b} \pm 0.05$	$0.24^{b} \pm 0.01$	$0.29^{b} \pm 0.02$	$0.66^{b} \pm 0.03$	$2169^{a} \pm 95$	812 ± 63	$323^{b} \pm 14$	$146^{b} \pm 5.8$	$2.24^{b} \pm 0.16$	$0.058^{b} \pm 0.01$
Ala-Gln + LPS	$7.70^{b} \pm 0.32$	$7.26^{b} \pm 0.37$	$1.38^{b} \pm 0.09$	$0.22^{b} \pm 0.02$	$0.26^{b} \pm 0.02$	$0.65^{b} \pm 0.04$	$2302^{a} \pm 80$	832 ± 59	$316^{b} \pm 20$	$152^{b} \pm 6.6$	$2.30^{b} \pm 0.18$	$0.060^{b} \pm 0.02$

Values are mean ± SEM, $n = 10$. Mean sharing different superscript letters within a column differ ($P < 0.05$). The jejunum and blood samples were obtained at 48 h after LPS injection (1 h after suckling and oral administration of water, Ala, Gln, or Ala-Gln)

Aden adenosine, *GSHR* ratios of oxidized glutathione to reduced glutathione (μmol/μmol)

Table 7 Protein levels of Toll-like receptor-4, active caspase-3, and NF*k*B in the jejunum of LPS-challenged piglets receiving oral administration of water, Ala, Gln or Ala-Gln

Treatment	TLR4	Caspase-3	NF*k*B
H_2O + saline	1.00 ± 0.09^{b}	1.00 ± 0.14^{b}	1.00 ± 0.18^{b}
H_2O + LPS	1.95 ± 0.27^{a}	6.32 ± 1.15^{a}	7.05 ± 1.64^{a}
Ala + LPS	1.82 ± 0.24^{a}	6.16 ± 1.28^{a}	7.24 ± 1.83^{a}
Gln + LPS	1.23 ± 0.18^{b}	1.26 ± 0.24^{b}	1.34 ± 0.29^{b}
Ala-Gln + LPS	1.16 ± 0.16^{b}	1.08 ± 0.21^{b}	1.27 ± 0.25^{b}

Values for the H_2O + saline group were normalized to 1.00 and are expressed as mean ± SEM, $n = 8$. Mean sharing different superscript letters within a column differ ($P < 0.01$). The jejunum was obtained at 24 h after LPS injection

amount of milk-born Gln was inadequate for maximal growth of healthy or endotoxin-challenged piglets, as oral administration of Gln greatly enhanced their daily weight gain (Table 4). The effect of Ala-Gln resulted from its Gln moiety, because Ala had no effect on either cell death in vitro (Table 2) or piglet intestinal morphology in vivo (Table 4).

Gln is transported across the intestinal plasma membrane primarily by N, LAT3 and ATB^{o}/ASCT2 systems (Bröer 2008). Dipeptides can be either directly taken up into enterocytes by H^{+}-driven PepT1 transporters or readily hydrolyzed by the plasma membrane-bound peptidases into individual free AA for subsequent transport (Fig. 3). Once entering enterocytes, Gln or Ala-Gln is extensively catabolized to generate ATP and many nitrogenous metabolites (Wu 1998). Rates of product formation in enterocytes were similar when cells were incubated with equal molar Gln and Ala-Gln. Thus, Ala-Gln is an excellent source of Gln for enterocytes, as reported for neutrophils (Mühling et al. 2007) and humans (Engel et al. 2008). Interestingly, some of the orally administered Gln was not degraded by the piglet small intestine in first-pass metabolism and entered the portal circulation (Table 4). Additionally, oral Ala-Gln increased concentrations of free Gln in both plasma and jejunal lumen to a great extent than oral Gln (Table 4). We speculate that Ala-Gln may be present in the small-intestinal lumen for a longer period of time than Gln for absorption and that some of the dipeptide may enter the portal circulation in vivo.

Products of Gln catabolism via glutaminase, glutamine:fructose-6-phosphate transaminase, and carbamoylphosphate synthase-II include glutamate, ornithine, glucosamine, and nucleic acids (Dekaney et al. 2008; Hu et al. 2008; Wu and Morris 1998). Glutamate is further degraded to generate aspartate and Ala (Wu et al. 2007). However, none of these Gln metabolites could prevent the H_2O_2- or LPS-induced enterocyte death. Likewise, blockage of Gln degradation by phosphate-activated glutaminase

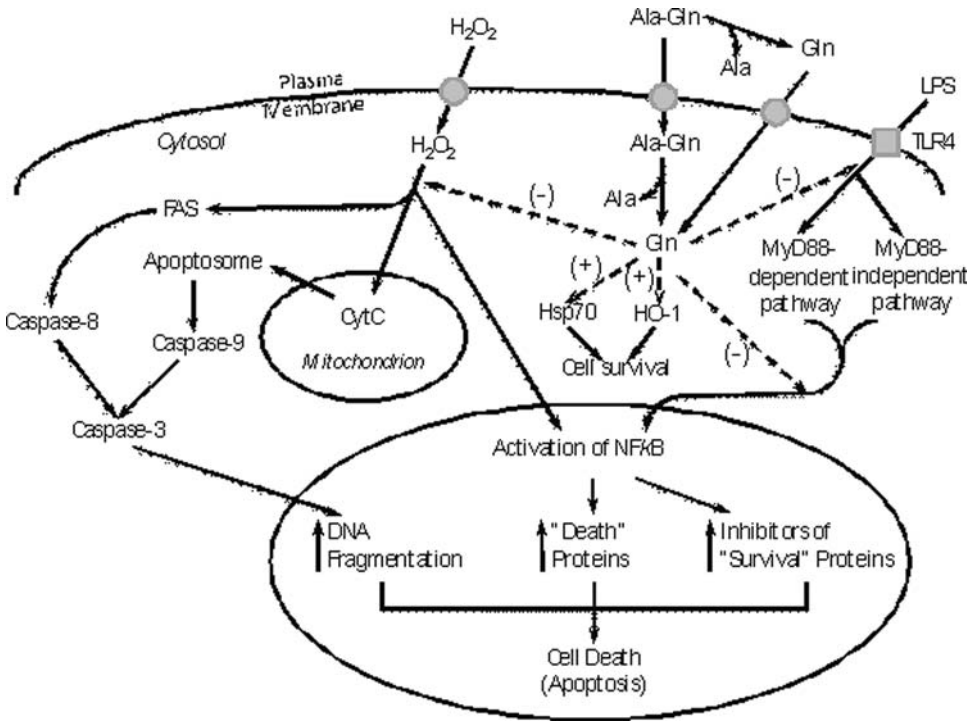

Fig. 3 Proposed mechanisms for the effect of Gln or Ala-Gln to prevent intestinal cells from oxidant- or LPS-induced apoptosis. *CytC* Cytochrome C, *hsp* heat shock protein, *HO-1* heme oxygenase-1

or inhibition of glutathione synthesis did not prevent the cytoprotective effect of Gln. Thus, it is possible that Gln exerts its effects on enterocytes by directly regulating cellular signaling pathways (e.g., mTOR and MAPK activation) and metabolism (Liao et al. 2008; Rhoads and Wu 2008). In support of this view, Coeffier et al. (2003) reported that enteral Gln stimulated protein synthesis in human gut mucosa. Furthermore, Gln increased intestinal expression of ornithine decarboxylase (Rhoads et al. 1997), a key enzyme in the synthesis of polyamines from ornithine (Flynn et al. 2008; Wu et al. 2008a).

Hydrogen peroxide is an oxidant to mammalian cells (Chang et al. 2008; Rider et al. 2007). However, it is unlikely that 0.5 mM H_2O_2 killed enterocytes directly through its oxidative effect because of the following reasons. First, the chemical property of H_2O_2 was not altered in the presence of Gln and yet Gln prevented the cytotoxic effect of H_2O_2 in a dose-dependent manner (Fig. 1). Second, the death of enterocytes did not occur immediately after exposure to H_2O_2 but required a prolonged period of time (e.g., 12 h) to affect gene expression and signaling events. It is now clear that H_2O_2 induces cell death through the activation of caspase-3 and NF*k*B, a master mediator of oxidative stress (Jones 2008; Wang et al. 2009). Because diffusion of H_2O_2 into cells is limited, it is transported across the plasma membrane by aquaporin homologues (Biener et al. 2006). Once inside cells, H_2O_2 activates Fas and enhances the release of cytochrome C from mitochondria, which ultimately results in the formation of caspase-3 via the caspase-8 and caspase-9 pathways (Fig. 3). Upon its generation, caspase-3 induces DNA fragmentation in cells (Jones 2008). Additionally, H_2O_2 activates NF*k*B, leading to increased expression of "death" proteins and inhibitors of "survival" proteins, and, therefore, apoptosis (Chandra et al. 2000).

The LPS-challenged piglet provides a good animal model to study the intestinal response to inflammatory pathogens (Li et al. 2007). Gln concentrations in plasma were reduced in LPS-challenged piglets (Table 5), as reported for infants with necrotizing enterocolitis (Becker et al. 2000), which likely results from increased catabolism of Gln in enterocytes, leukocytes, and other intestinal cells of the intestinal mucosa (Lagranha et al. 2008a; Mühling et al. 2007; Wu et al. 1995). LPS binds to Toll-like receptor 4 (TLR4) on the plasma membrane, which triggers the MyD88-dependent and independent pathways, resulting in activation of NF*k*B and apoptosis (Gribar et al. 2008; Lu et al. 2008). Interestingly, oral administration of Gln or Ala-Gln to sow-reared piglets reduced LPS-induced fever and prevented intestinal injury (Table 4). Similarly, Gln treatment ameliorated gut damage in endotoxin-infected rats (Kessel et al. 2008; Uehara et al. 2005). Three mechanisms may be coordinately responsible for the anti-apoptotic effect of Gln in enterocytes. First, Gln inhibits intestinal expression of TLR4 (Table 7) and MyD88 (Kessel et al. 2008), as well as NF*k*B activation (Table 7) in H_2O_2- or LPS-challenged mucosal cells. Second, Gln attenuates the production of active caspase-3 (Table 7) through (1) a cytochrome C-dependent mechanism and (2) inhibition of nitric-oxide

synthesis by inducible nitric-oxide synthase (Umeda et al. 2009; Wu and Meininger 2002), therefore preventing DNA damage as well as protein oxidation and *S*-nitrosylation (Galli 2007; Mannick 2007; Voss and Grune 2007). Third, Gln increases intestinal expression of antioxidative proteins, including glutathione transferase-ω (Wang et al. 2008b), heme oxygenase-1 (Uehara et al. 2005) [a master regulator of oxidative defense and cell homeostasis (Muz et al. 2008; Olszanecki et al. 2008)], and heat-shock proteins [cytoprotective proteins; (Madden et al. 2008; Wischmeyer et al. 1997)], therefore providing a cytoprotective effect. Gln or Ala-Gln is likely beneficial for treating sepsis and other inflammatory bowel disease in neonates.

In conclusion, our results indicate that oral Gln or Ala-Gln increases Gln concentrations in the intestinal tract and plasma, enhances small-intestinal and whole-body growth performance, and provides effective protection from oxidant- or endotoxin-induced death of neonatal enterocytes. Our findings support supplementation of Gln or Ala-Gln to current Gln-free pediatric AA solutions to prevent intestinal oxidative injury and inflammatory disease in neonates.

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