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ORIGINAL ARTICLE

Protective effects of *N*-acetylcysteine on intestinal functions of piglets challenged with lipopolysaccharide

Yongqing Hou · Lei Wang · Wei Zhang · Zhenguo Yang · Binying Ding · Huiling Zhu · Yulan Liu · Yinsheng Qiu · Yulong Yin · Guoyao Wu

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Abstract The neonatal small intestine is susceptible to damage by endotoxin, but effective methods for prevention and treatment are lacking. *N*-acetylcysteine (NAC) is a widely used precursor of L-cysteine for animal cells and plays an important role in protecting cells against oxidative stress. This study was conducted with the lipopolysaccharide (LPS)-challenged piglet model to determine the effects of NAC on intestinal function. Eighteen piglets were randomly allocated into control, LPS and LPS + NAC groups. The control and LPS groups were fed a corn- and soybean meal-based diet, and the LPS + NAC group was fed the basal diet +500 mg/kg NAC. On days 10, 13 and 20 of the trial, the LPS and LPS + NAC groups received intraperitoneal administration of LPS (100 μg/kg BW), whereas the control piglets received saline. On day 20 of the trial, D-xylose (0.1 g/kg BW) was orally administrated to all piglets 2 h after LPS or saline injection, and blood samples were collected 1 h thereafter. One hour blood xylose test was used to measure intestinal absorption capacity and mucosal integrity, and diamine oxidase (DAO) was used as a marker of intestinal injury. On day 21 of the trial, pigs were killed to obtain the intestinal mucosa. Compared to the control, LPS challenge reduced ($P < 0.05$) the concentrations of D-xylose (a marker of intestinal absorption) in plasma, activities of DAO in the jejunal mucosa, the ratio of villus height to crypt depth in the jejunal mucosa, RNA/DNA and protein/DNA in the jejunal and ileal mucosae, while increasing ($P < 0.05$) DAO activity in plasma and caspase-3 expression in the intestinal mucosa. The adverse effects of LPS were partially ameliorated ($P < 0.05$) by NAC supplementation. Moreover, NAC prevented the LPS-induced decrease in claudin-1 and occludin expression in the jejunal and ileal mucosae. Collectively, these results indicate that dietary NAC supplementation alleviates the mucosal damage and improves the absorptive function of the small intestine in LPS-challenged piglets.

Keywords *N*-acetylcysteine · Intestinal functions · Piglets · Lipopolysaccharide

Abbreviations

BSA	Bovine serum albumin
BW	Body weight
DAO	Diamine oxidase
HSP 70	Heat shock protein 70
LPS	Lipopolysaccharide
NAC	*N*-acetylcysteine
PBS	Phosphate-buffered saline
SD	Standard deviation of the mean

Y. Hou (✉) · L. Wang · W. Zhang · Z. Yang · B. Ding · H. Zhu · Y. Liu · Y. Qiu
Hubei Key Laboratory of Animal Nutrition and Feed Science, Wuhan Polytechnic University, Wuhan 430023, China
e-mail: houyq777@yahoo.com.cn

Y. Yin
Research Center for Healthy Breeding of Livestock and Poultry, Hunan Engineering and Research Center of Animal and Poultry Science and Key Laboratory for Agro-ecological Processes in Subtropical Region, Institute of Subtropical Agriculture, The Chinese Academy of Sciences, Hunan 410125, China

G. Wu (✉)
Department of Animal Science, Texas A & M University, College Station, TX 77843, USA
e-mail: g-wu@tamu.edu

Introduction

N-acetylcysteine (NAC) is an effective precursor of L-Cysteine for animal cells and, therefore, plays an important role in protecting cells against oxidative stress (Wu et al. 2004). NAC is produced in living organisms from cysteine and is contained in natural foods (Knight et al. 1991; De Flora et al. 1991). In neonates, the synthesis of cysteine is limited (Wu 2009), indicating that its endogenous provision is negligible in vivo. Indeed, NAC cannot be detected in pigs without its supplementation, which necessitates oral administration to improve antioxidative function under inflammatory conditions.

The neonatal small intestine is susceptible to damage by endotoxin, but effective methods for prevention and treatment are currently lacking (Hou et al. 2011a, b). Lipopolysaccharide (LPS) results in a variety of morphologic alterations in the digestive tract and an increase in mucosal permeability (Hou et al. 2010; 2011a, b; Liu et al. 2008). Moreover, LPS treatment greatly increases HSP70 expression in the pig small intestine (Hou et al. 2010), whereas a high level of HSP70 is indicative of oxidative stress in intestinal cells (Hou et al. 2010; Sepponen and Poso 2006). Orally administered NAC is very rapidly utilized by the intestine to produce glutathione (Hagen et al. 1990; Vincenzini et al. 1988). Accordingly, dietary supplementation with NAC may improve intestinal morphology and function of piglets in response to endotoxin. At present, little is known about the effects of NAC on the small intestine under LPS challenge. We hypothesized that NAC may improve mucosal function in the small intestine of LPS-challenged piglets, which is a well-characterized animal model for studying infant nutrition and physiology (Blachier et al. 2009, 2010; Wu et al. 2010, 2011; Yin et al. 2010). The purpose of the present study was to test this hypothesis and to elucidate the underlying molecular mechanisms.

Materials and methods

Animal care and diets

The animal use protocol for this research was approved by the Animal Care and Use Committee of Hubei Province. All pigs used in this experiment were born naturally at term (114 days of gestation). Eighteen crossbred healthy female piglets (Duroc × Landrace × Yorkshire) were reared by sows and weaned at 28 ± 2 days of age. After a 7-day period of adaptation, the pigs (35 ± 2 days of age, average body weight of 11.58 ± 0.26 kg) were housed individually in stainless steel metabolic cages (1.20 × 1.10 m^2) and maintained at an ambient temperature of 22–25°C in an environmentally controlled room by air-conditioning (Hou et al. 2010). Each cage was equipped with a feeder and a nipple waterer to allow piglets free access to food and drinking water (Hou et al. 2010, 2011a, b; Tan et al. 2009a). The corn- and soybean meal-type basal diet (Table 1) was formulated to meet the National Research Council (NRC 1998) requirements for all nutrients, including amino acids (Li et al. 2011b).

Table 1 Composition and nutrient contents of the basal diet (air-dry basis)

Items	Content
Ingredients (%)	
Corn	61.88
Soybean meal	21.98
Wheat middling	4.00
Fish meal	3.00
Dried whey	3.00
Soy protein concentrate	1.50
$CaHPO_4$	1.25
Premix[a]	1.00
Limestone	0.69
Soy oil	0.50
Acidifier	0.30
NaCl	0.30
Mold inhibitor	0.10
Choline chloride	0.20
L-Lysine·HCl (78.8% lysine)	0.25
DL-Methionine (99% methionine)	0.05
Nutrients composition	
Digestible energy[b] (M J/kg)	14.22
Crude protein (%)[c]	20.90
Total lysine (%)[b]	1.15
Total methionine (%)[b]	0.30
Total methionine + cystine (%)[b]	0.65
Total threonine (%)[b]	0.74
Total tryptophan (%)[b]	0.21
Calcium (%)[c]	0.70
Total phosphorus (%)[c]	0.60
Available phosphorus (%)[b]	0.32

[a] Premix provided the following amounts of vitamins and trace minerals per kilogram of the complete diet: Fe, 100 mg ($FeSO_4$ H_2O); Cu, 150 mg ($CuSO_4$ $5H_2O$); Mn, 40 mg ($MnSO_4$ $5H_2O$); Zn, 100 mg ($ZnSO_4$ $7H_2O$); I, 0.5 mg (KI); Se, 0.3 mg (Na_2SeO_3 $5H_2O$); retinol acetate, 10,800 IU; cholecalciferol, 4,000 IU; dl-α-tocopheryl acetate, 40 IU; vitamin K_3, 4 mg; thiamin, 6 mg; riboflavin, 12 mg; pyridoxine, 6 mg; vitamin B_{12}, 0.05 mg; biotin, 0.2 mg; folic acid, 2 mg; niacin, 50 mg; D-calcium pantothenate, 25 mg

[b] Calculated value

[c] Analyzed value

Experimental design

All piglets had free access to the basal diet (Table 1) between days 28 and 35 of age (days 0–7 postweaning) for adapting to solid food. At 35 days of age, piglets were assigned randomly into one of three groups: (1) Control group (piglets fed the basal diet and receiving intraperitoneal administration of sterile saline); (2) LPS group (piglets fed the basal diet and receiving intraperitoneal administration of *Escherichia coli* LPS); (3) LPS + NAC group (piglets fed the basal diet supplemented with 500 mg/kg NAC and receiving intraperitoneal administration of LPS). LPS was dissolved in saline. NAC (powder) was well mixed with the basal diet. The control and LPS diets were supplemented with 500 mg/kg cornstarch, respectively, to obtain approximately isocaloric diets. The dosage of 500 mg/kg NAC was chosen according to our previous findings that dietary supplementation with 500 mg/kg NAC could relieve the growth depression in weanling piglets challenged by LPS (Hu et al. 2010). Because the supplementation with 500 mg/kg NAC resulted in only an increase of 0.0042% nitrogen, we deemed it not necessary to use non-essential amino acids as isonitrogenous control. There were six piglets per group. On days 10, 13 and 20 of the trial, overnight fasted piglets in the LPS and LPS + NAC groups received intraperitoneal administration of LPS (*Escherichia coli* serotype 055: B5; Sigma Chemical Inc., St. Louis, MO, USA) at the dose of 100 μg/kg BW, whereas piglets in the control group received intraperitoneal administration of the same volume of saline. The dosage of LPS was chosen according to our previous studies (Hu et al. 2010; Liu et al. 2008). During days 0–10 of the trial (pre-challenge), all the piglets had free access to feed and drinking water. To exclude a possible effect of LPS-induced reduction in food intake on the piglet intestine, the control and LPS + NAC piglets were pair-fed during days 10–16 of the trial (post-challenge with LPS) the same amount of feed per kg body weight as LPS piglets. On day 20, D-xylose was orally administrated to piglets at the dose of 0.1 g/kg BW (infused with 10% D-xylose at 1 mL/kg BW); 2 h after LPS or saline administration, D-xylose absorptive test was used to measure intestinal absorption capacity and mucosal integrity (Haeney et al.1978). At 24 h after the administration of LPS or saline (on day 21), all pigs were killed during sodium pentobarbital anesthesia (50 mg/kg, iv) to obtain the small intestine (Hou et al. 2010, 2011a, b; Deng et al. 2009).

Blood sample collection

On day 20 of the trial, 1 h after infusion of D-xylose (3 h after LPS or saline administration), blood samples were collected from the anterior vena cava into heparinized vacuum tubes (Becton–Dickinson Vacutainer System, Franklin Lake, NJ, USA), as described (Hou et al. 2010). Blood samples were centrifuged at 3,000 rpm for 10 min at 4°C to obtain plasma (Hou et al. 2010; Tan et al. 2009b). Plasma was stored at −80°C until analysis.

Intestinal sample collection

The pig abdomen was opened immediately from the sternum to the pubis, and the whole gastrointestinal tract was immediately exposed (Hou et al. 2010; Li et al. 2009). The small intestine was dissected free of the mesentery and placed on a chilled stainless steel tray. The 5 and 10 cm segments were cut at the distal duodenum, mid-jejunum and mid-ileum, respectively (Hou et al. 2010; Wang et al. 2008). The 5 cm intestinal segments were flushed gently with ice-cold phosphate-buffered saline (PBS, pH 7.4) and then placed in 10% fresh, chilled formalin solution for histological measurements (Hou et al. 2010; Nofrarías et al. 2006).

Intestinal segments (10 cm in length) were opened longitudinally and the contents were flushed with ice-cold PBS (Hou et al. 2010; Wang et al. 2008). Mucosa was collected by scraping using a sterile glass microscope slide at 4°C (Hou et al. 2010; Wang et al. 2009), rapidly frozen in liquid nitrogen and stored at −80°C until analysis. All samples were collected within 20 min after being killed.

Intestinal morphology

Intestinal segments for morphological analysis were dehydrated and embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin (Hou et al. 2010; Luna 1968). Morphological measurements were performed with a light microscope (American Optical Co., Scientific Instrument Div., Buffalo, NY, USA). Villus height (the distance from the villus tip to the crypt mouth) and width (the distance of the widest villi), crypt depth (the distance from the crypt mouth to the base) were measured using a linear ocular micrometer with a computer-assisted morphometric system (BioScan Optimetric, BioScan Inc., Edmonds, WA, USA). Only vertically oriented villi and crypts were measured (Hou et al. 2010; Nofrarías et al. 2006; Wu et al. 1996; Uni et al. 1998). Values are expressed as means from ten adjacent villi. The villus: crypt ratio and villous surface area (VSA) were calculated.

Determination of D-xylose in plasma

D-Xylose in plasma was determined as described by Hou et al. (2010). Briefly, 50 μL of the collected plasma was added to 5 mL of the phloroglucinol color reagent solution (Sigma Chemical Inc., St. Louis, MO, USA) and heated at

100°C for 4 min. The samples were allowed to cool to room temperature in a water bath. A xylose standard solution was prepared by dissolving D-xylose in saturated benzoic acid (prepared in deionized water) to obtain 0, 0.7, 1.3 and 2.6 mmol/L. The xylose standard solution or the sample was added to the color reagent solution. Absorbance of the resultant mixture at 554 nm was measured using a spectrophotometer (Model 6100, Jenway LTD., Felsted, Dunmow, CM6 3LB, Essex, England, UK). The standard solution of 0 mmol/L D-xylose was considered as the blank.

Determination of diamine oxidase (DAO) activity in plasma and intestinal mucosa

Diamine oxidase (DAO) activities in plasma and small intestinal mucosa were determined using spectrophotometry as described by Hosoda et al. (1989). For measurement of intestinal DAO activity, the small intestinal mucosa (500 mg) was homogenized in a threefold volume of phosphate buffer (0.1 M, pH 7.2) and centrifuged at 10,000×*g* for 30 min at 4°C. The supernatant fluid was used for DAO assays (Li et al. 1996). The assay mixture (3.8 mL) contained 3 mL of phosphate buffer (0.2 M, pH 7.2), 0.1 mL (0.004%) of horseradish peroxidase solution (Sigma Chemicals), 0.1 mL of o-dianisidine–methanol solution [0.5% of *o*-dianisidine (Sigma Chemicals) in methanol], 0.5 mL of plasma or intestinal homogenate and 0.1 mL of substrate solution (0.175% of cadaverine dihydrochloride, Sigma Chemicals). This mixture was incubated for 30 min at 37°C, and absorbance at 436 nm was measured to indicate DAO activity (Hou et al. 2011a). Protein was measured as described by Hou et al. (2010). DAO was used as a marker of intestinal injury (Luk et al. 1980).

Measurement of mucosal DNA, RNA and protein

The DNA, RNA and protein were extracted from the small intestinal mucosa, using TRI REAGENT-RNA/DNA/Protein isolation reagent and their concentrations were determined colorimetrically (Liu et al. 2010; Hou et al. 2010). Intestinal mucosal DNA was analyzed fluorimetrically using the method of Prasad et al. (1972). RNA was determined by spectrophotometry using a modified Schmidt-Tannhauser method as described by Munro and Fleck (1969). Protein was analyzed according to the method of Lowry et al. (1951). For measurement of small intestinal DNA and RNA levels, the mucosa was homogenized (~2 min) in a 100-fold volume of ice-cold saline (0.9%) and the homogenate was centrifuged at 1,800×*g* for 10 min at 4°C to obtain the supernatant fluid for analysis. For measurement of mucosal protein, intestinal mucosal samples (~0.1 g) were homogenized with a tissue homogenizer in 1 mL of ice-cold PBS-EDTA buffer (0.05 mol/L Na_3PO_4, 2.0 mol/L NaCl, 2 mmol/L EDTA, pH 7.4) and the homogenates were centrifuged at 12,000×*g* for 10 min at 4°C to obtain the supernatant fluid for assays.

Protein immunoblot analysis

Analysis of caspase-3, claudin-1 and occludin proteins were performed by Western blotting as described by Hou et al. (2010). Briefly, frozen intestinal mucosal samples were powdered under liquid nitrogen using a mortar and pestle. The powdered samples (100 mg) were homogenized in 1 mL of lysis buffer with a Polytron homogenizer. The homogenates were centrifuged at 12,000×*g* for 15 min at 4°C. The supernatant fluid was aliquoted into microcentrifuge tubes, to which 2× SDS sample buffer (2 mL of 0.5 mol/L Tris, pH 6.8, 2 mL glycerol, 2 mL of 10% SDS, 0.2 mL of β-mercaptoethanol, 0.4 mL of a 4% solution of bromophenol blue and 1.4 mL of water) was added in a 1:1 ratio. The samples were boiled for 5 min and cooled on ice before being used for Western blot analysis. Proteins (150 μg/sample for caspase-3; 60 μg/sample for claudin-1, β-actin and GAPDH; 120 μg/sample for occludin) were separated by electrophoresis on a 10% (for caspase-3, occludin, β-actin and GAPDH) or 12% (for claudin-1) polyacrylamide gel. Proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. Non-fat dry milk in TBS/T buffer was used to block filters for at least 1 h at room temperature. Membranes were incubated with primary antibodies overnight at 4°C: caspase-3 (rabbit polyclonal antibodies from Cell Signaling Technology, Inc., Danvers, MA, USA; dilution 1:1,000), claudin-1 (rabbit monoclonal antibodies from Invitrogen Technology, Inc., Danvers, MA, USA; dilution 1:1,000), occludin (mouse monoclonal antibodies from Invitrogen Technology; dilution 1:1,000), GAPDH (HRP-labeled GAPDH, Shanghai Kangcheng Company, China; dilution 1:10,000) or β-actin (mouse monoclonal antibody from Sigma Chemicals; dilution 1:5,000). The primary antibody dilution buffer was 1× TBS and 0.1% Tween-20 with 5% BSA. The membranes were washed three times with TBS-T (1× Tris-buffered saline including 0.1% Tween 20) and incubated for 1 h at room temperature with anti-rabbit (mouse) immunoglobulin G horseradish peroxidase-conjugated secondary antibody (Beijing ZhongShan Golden Bridge Biological Technology Co., LTD, China; dissolved in 5% non-fat dry milk in TBS-Tween-20 buffer in 1:5,000 dilution). Incubation of primary antibodies was followed by three washes with TBS-T buffer for 10 min, and incubation of the secondary antibodies was followed by five washes for 8 min. Blots were developed using an enhanced

chemiluminescence Western blotting kit (ECL-plus, Amersham Biosciences, Sweden), visualized using a Gene Genome bioimaging system and analyzed using GeneTools software (Syngene, Frederick, MD, USA).

Statistical analysis

Results are expressed as mean ± SD and analyzed by one-way analysis of variance (ANOVA). Differences among treatment means were determined by the Duncan's multiple range test (Wei et al. 2011). All statistical analyses were performed using SPSS 13.0 software (SPSS Inc. Chicago, IL, USA). Probability values ≤ 0.05 were taken to indicate significance.

Results

Feed intake and average daily weight gain of piglets

Between days 10 and 20 of the trial (LPS challenge period), average feed intake of piglets did not differ among the control, LPS and LPS + NAC groups. LPS administration reduced ($P < 0.05$) daily weight gains of piglets, and dietary supplementation of NAC increased ($P < 0.05$) the daily weight gains of LPS-challenged pigs (Table 2).

Intestinal morphology

Data on the small intestinal morphology are summarized in Table 3. Compared to control, LPS piglets exhibited an increase ($P < 0.05$) in crypt depth in the duodenum and jejunum, as well as reductions ($P < 0.05$) in villus height, villous surface area in the jejunum and ileum, and the ratio of villus height to crypt depth in the duodenum and jejunum. In comparison with the LPS piglets, dietary supplementation of 500 mg/kg NAC decreased ($P < 0.05$) crypt depth in the jejunum, while increasing ($P < 0.05$) villus height in the ileum, villous surface area and the ratio of villus height to crypt depth in the jejunum.

Concentrations of plasma D-xylose, diamine oxidase (DAO) activity in plasma and intestinal mucosa

Data on plasma D-xylose, diamine oxidase (DAO) activities in plasma and intestinal mucosa are summarized in Table 4. Compared with the control piglets, LPS piglets exhibited a decrease ($P < 0.05$) in D-xylose concentrations in plasma and DAO activity in the jejunum, and an increase ($P < 0.05$) in DAO activity in the plasma. In comparison with the LPS piglets, dietary supplementation of 500 mg/kg NAC increased ($P < 0.05$) D-xylose concentrations in plasma and DAO activity in the jejunum, while decreasing ($P < 0.05$) DAO activity in the plasma.

Concentrations of DNA, RNA and protein in the small intestinal mucosa

Compared to the control, LPS challenge reduced DNA concentrations in the jejunum and ileum, RNA/DNA ratio in the duodenum, jejunum and ileum, and protein/DNA ratio in the jejunum and ileum. In comparison with the LPS group, dietary supplementation of 500 mg/kg NAC increased ($P < 0.05$) DNA concentrations in the jejunum, and RNA/DNA and protein/DNA ratio in the jejunum and ileum (Table 5).

Abundance of caspase-3 protein in the small intestinal mucosa

LPS administration increased ($P < 0.05$) the abundance of caspase-3 protein in the small intestinal mucosa, compared to control piglets. Relative to LPS piglets, dietary supplementation with 500 mg/kg NAC decreased ($P < 0.05$) caspase-3 expression in the intestinal mucosa (Fig. 1).

Abundance of claudin-1 and occludin proteins in the small intestinal mucosa

LPS administration decreased ($P < 0.05$) the abundance of claudin-1 and occludin proteins in the duodenal, jejunal and ileal mucosae, compared to the control. Dietary

Table 2 Effects of dietary NAC supplementation on growth performance of piglets after LPS challenge

Items	Control	LPS	LPS + NAC
Body weight at day 10 of the trial (kg)	15.4 ± 0.55	15.7 ± 0.54	16.0 ± 0.63
Average daily feed intake between days 10 and 20 of the trial (g/day)	657 ± 60.3	664 ± 58.6	667 ± 61.2
Average daily weight gain between days 10 and 20 of the trial (g/day)	310 ± 12.3[a]	269 ± 11.8[b]	289 ± 13.0[a]

Data are mean ± SD, $n = 6$. Control (non-challenged control), piglets fed the basal diet and injected with sterile saline; LPS (LPS-challenged control), piglets fed the basal diet and challenged with *Escherichia coli* LPS; LPS + NAC (LPS + 500 mg/kg NAC), piglets fed the basal diet supplemented with 500 mg/kg and challenged with LPS

[a,b] Values within a row with different letters differ ($P < 0.05$)

Table 3 Effects of NAC supplementation on the intestinal mucosal morphology of pigs after LPS challenge

Items	Control	LPS	LPS + NAC
Villus height (μm)			
Duodenum	388.17 ± 14.64	376.83 ± 9.39	369.75 ± 17.26
Jejunum	421.15 ± 11.23[a]	399.53 ± 12.78[b]	406.11 ± 8.24[b]
Ileum	375.43 ± 17.23[a]	349.34 ± 13.80[b]	373.68 ± 12.68[a]
Crypt depth (μm)			
Duodenum	102.98 ± 5.23[b]	118.81 ± 7.08[a]	113.28 ± 7.87[a]
Jejunum	111.70 ± 6.86[b]	122.81 ± 7.12[a]	113.18 ± 7.41[b]
Ileum	114.34 ± 10.16	117.10 ± 9.96	112.59 ± 5.03
Villus height/crypt depth			
Duodenum	3.78 ± 0.21[a]	3.18 ± 0.13[b]	3.28 ± 0.25[b]
Jejunum	3.78 ± 0.17[a]	3.26 ± 0.21[b]	3.61 ± 0.29[a]
Ileum	3.31 ± 0.40	3.01 ± 0.32	3.32 ± 0.13
Villous surface area (cm^2)			
Duodenum	5.08 ± 0.28	4.81 ± 0.47	5.03 ± 0.27
Jejunum	5.61 ± 0.39[a]	4.69 ± 0.25[c]	5.14 ± 0.29[b]
Ileum	5.28 ± 0.35[a]	4.67 ± 0.32[b]	4.96 ± 0.39[ab]

Data are mean ± SD, $n = 6$. Control (non-challenged control), piglets fed the basal diet and injected with saline; LPS (LPS-challenged control), piglets fed the basal diet and challenged with *Escherichia coli* LPS; LPS + NAC (LPS + 500 mg/kg NAC), piglets fed the basal diet supplemented with 500 mg/kg and challenged with LPS. Villous surface area = $2\pi rh$ (r = villus width/2, h = villus height)

[a,b,c] Values within a row with different letters differ ($P < 0.05$)

Table 4 Effects of dietary NAC supplementation on D-xylose concentrations in plasma, the activity of DAO in plasma and intestinal mucosa of piglets after LPS challenge

Items	Control	LPS	LPS + NAC
D-xylose in plasma (μg/mL)	0.590 ± 0.100[a]	0.409 ± 0.029[b]	0.510 ± 0.049[a]
DAO in plasma (U/mL)	6.974 ± 0.682[b]	8.684 ± 0.933[a]	7.584 ± 0.598[b]
DAO in duodenum (U/mg protein)	0.278 ± 0.060[a]	0.190 ± 0.064[b]	0.172 ± 0.047[b]
DAO in jejunum (U/mg protein)	0.199 ± 0.033[a]	0.120 ± 0.027[b]	0.165 ± 0.021[a]
DAO in ileum (U/mg protein)	0.061 ± 0.023	0.042 ± 0.022	0.054 ± 0.012

Data are mean ± SD, $n = 6$. Control (non-challenged control), piglets fed the basal diet and injected with saline; LPS (LPS-challenged control), piglets fed the basal diet and challenged with *Escherichia coli* LPS; LPS + NAC (LPS + 500 mg/kg NAC), piglets fed the basal diet supplemented with 500 mg/kg and challenged with LPS

[a,b] Values within a row with different letters differ ($P < 0.05$)

supplementation with 500 mg/kg NAC increased ($P < 0.05$) claudin-1 expression in the duodenal, jejunal and ileal mucosae, and occludin expression in the ileal mucosa, in comparison with the LPS group (Fig. 2, 3).

Discussion

NAC is a chemically stable precursor of cysteine for glutathione synthesis (Wu et al. 2004). The results of many studies have demonstrated that glutathione and its sulfur-containing precursors are important in maintaining the immune system in a healthy state, while protecting the organisms against cancer and heart disease (Hagen et al. 1990; Vincenzini et al. 1988). Similarly, NAC has been reported to attenuate inflammation in the liver of LPS-treated mice (Li et al. 2002a). Thus, NAC is commonly used in clinical toxicology for the treatment of acetaminophen poisoning. The LPS-challenged piglet provides a good animal model to study the intestinal response to inflammatory pathogens (Hou et al. 2010; Haynes et al. 2009; Li et al. 2007). It is noteworthy that recent studies have shown that dietary supplementation with 500 mg/kg NAC could increase the circulating levels of epidermal growth factor and relieve growth depression in weanling piglets that were challenged by LPS (Hu et al. 2010).

Standard physiological and molecular biology techniques were (Geng et al. 2011; Li et al. 2011a; Yao et al. 2011) employed in the present study to determine the response of piglets to endotoxin administration. LPS is known to induce a variety of morphologic alterations in the digestive tract (e.g., submucosal edema, epithelial lifting at the tips of the villi, reduced villus height and increased crypt depth in the mucosa) and an increase in mucosal permeability (Hou et al. 2010, 2011a, b; Liu et al. 2008).

Table 5 Effects of NAC supplementation on intestinal mucosal growth of piglets challenged with LPS

Items	Control	LPS	LPS + NAC
DNA (mg/g)			
Duodenum	0.373 ± 0.028	0.354 ± 0.039	0.363 ± 0.015
Jejunum	0.317 ± 0.254[a]	0.217 ± 0.026[b]	0.283 ± 0.030[a]
Ileum	0.540 ± 0.028[a]	0.408 ± 0.025[b]	0.430 ± 0.018[b]
RNA/DNA			
Duodenum	5.365 ± 0.430[a]	4.280 ± 0.364[b]	4.579 ± 0.571[a,b]
Jejunum	7.124 ± 1.768[a]	4.835 ± 0.382[c]	6.743 ± 1.634[b]
Ileum	4.544 ± 0.654[a]	2.644 ± 0.223[b]	3.960 ± 0.449[a]
Total protein/DNA			
Duodenum	165.97 ± 22.76	141.46 ± 23.35	155.17 ± 26.05
Jejunum	333.22 ± 44.71[a]	199.46 ± 27.87[c]	268.16 ± 35.28[b]
Ileum	175.39 ± 15.18[a]	115.56 ± 8.65[b]	158.53 ± 12.87[a]

Data are mean ± SD, $n = 6$. Control (non-challenged control), piglets fed the basal diet and injected with saline; LPS (LPS-challenged control), piglets fed the basal diet and challenged with *Escherichia coli* LPS; LPS + NAC (LPS + 500 mg/kg NAC), piglets fed the basal diet supplemented with 500 mg/kg and challenged with LPS

[a,b] Values within a row with different letters differ ($P < 0.05$)

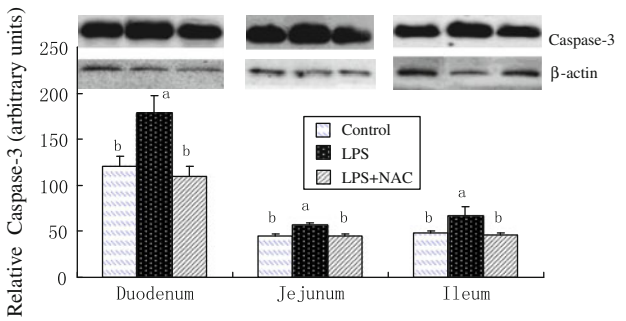

Fig. 1 Relative levels of the caspase-3 protein in the small intestinal mucosa of piglets. Mucosal extracts (150 μg protein/sample) were separated by 10% SDS–polyacrylamide gel electrophoresis for determination of caspase-3 and *β*-actin. Values for the caspase-3 protein were normalized for *β*-actin. Data are mean ± SD, $n = 6$. Control (non-challenged control), piglets fed the basal diet and injected with sterile saline; LPS (LPS-challenged control), piglets fed the basal diet and challenged with *Escherichia coli* LPS; LPS + NAC (LPS + 500 mg/kg NAC), piglets fed the basal diet supplemented with 500 mg/kg and challenged with LPS. *a, b* within the same intestinal segment, means with different superscripts differ ($P < 0.05$)

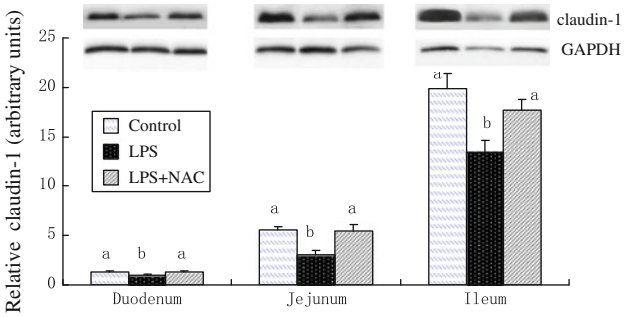

Fig. 2 Relative levels of the claudin-1 protein in the small intestinal mucosa of piglets. Mucosal extracts (60 μg protein/sample) were separated by 12% SDS–polyacrylamide gel electrophoresis for determination of claudin-1 and GAPDH. Values for relative claudin-1 were normalized for GAPDH. Data are mean ± SD, $n = 6$. Control (non-challenged control), piglets fed the basal diet and injected with saline; LPS (LPS-challenged control), piglets fed the basal diet and challenged with *Escherichia coli* LPS; LPS + NAC (LPS + 500 mg/kg NAC), piglets fed the basal diet supplemented with 500 mg/kg and challenged with LPS. *a, b* within the same intestinal segment, means with different superscripts differ ($P < 0.05$)

Additional indicators of the small intestinal morphology are villus height, crypt depth, the ratio of villus height to crypt depth, and villous surface area. Thus, an increase in villus height, villous surface area or villus/crypt ratio corresponds to improvement in the digestion and absorption of nutrients (Hou et al. 2010; Hampson 1986; Montagne et al. 2003). In this regard, it is noteworthy that NAC supplementation resulted in: (1) increased villus height; (2) decreased crypt depth; (3) an increased ratio of villus height to crypt depth; and (4) increased villous surface area (Table 3; Fig. 1). These findings support the notion that NAC beneficially alleviates the LPS-induced damage of the intestinal structure. Consistent with this view, dietary supplementation with 500 mg/kg NAC augmented the entry of orally administered D-xylose into the systemic circulation in LPS-challenged piglets (Table 4), which is a simple, specific and sensitive measure of intestinal absorption capacity (Hou et al. 2010).

In healthy pigs, D-xylose is readily absorbed by the small intestines. However, under conditions of malabsorption, the entry of D-xylose from the intestinal lumen to the portal vein is impaired, thereby explaining reduced concentrations in both blood and urine (Mansoori et al. 2009). Additionally, NAC enhanced DAO activity in the small intestinal mucosa and decreased DAO activity in plasma in response to LPS administration (Table 4). The

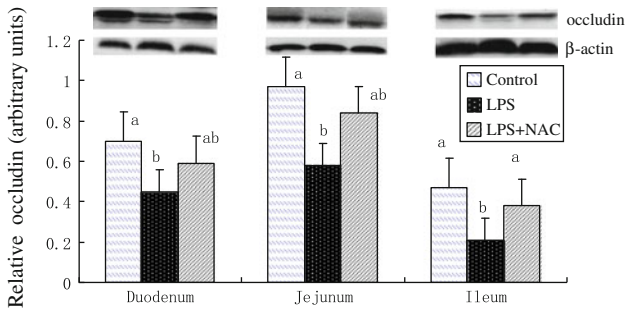

Fig. 3 Relative levels of the occludin protein in the small intestinal mucosa of piglets. Mucosal extracts (120 μg protein/sample) were separated by 10% SDS–polyacrylamide gel electrophoresis for determination of occludin and *β*-actin. Values for relative occludin were normalized for *β*-actin. Data are mean ± SD, $n = 6$. Control (non-challenged control), piglets fed the basal diet and injected with saline; LPS (LPS-challenged control), piglets fed the basal diet and challenged with *Escherichia coli* LPS; LPS + NAC (LPS + 500 mg/kg NAC), piglets fed the basal diet supplemented with 500 mg/kg and challenged with LPS. *a, b* Within the same intestinal segment, means with different superscripts differ ($P < 0.05$)

activity of the mucosal enzyme DAO can serve as a marker of intestinal mucosal maturation and integrity, and of mucosal injury and recovery. In addition, plasma DAO is a useful indicator of the severity of mucosal injury (Luk et al. 1983; Hou et al. 2011b). In mammals, DAO is abundantly expressed in the upper part of the intestinal mucosa, and therefore DAO activity is a noninvasive marker of alterations in intestinal mucosal function and structure (Li et al. 1996). Under certain circumstances, intestinal mucosal cells underwent necrosis and sloughed off into the intestinal lumen, leading to a decrease in intestinal mucosal DAO and an increase in circulating levels of DAO (Li et al. 2002b). Taken together, these data indicate that NAC can enhance the ability of the small intestine to absorb nutrients and barrier function under inflammatory conditions.

Intestinal biochemical indices, such as DNA concentrations, as well as RNA/DNA and protein/DNA ratios, can be used to assess intestinal development (Fasina et al. 2007; Iji et al. 2001; Jeurissen et al. 2002; Uni et al. 1998). DNA concentration reflects the rate of mitosis for producing new columnar epithelial cells, RNA/DNA ratios indicate cellular efficiency, and protein/DNA ratios implicate the efficiency of protein synthesis in cells (Fasina et al. 2007). In the present study, dietary supplementation with NAC attenuated the decrease of DNA concentration, as well as RNA/DNA and protein/DNA ratios in the jejunal mucosa of LPS-challenged piglets (Table 5). The results of these studies clearly reveal that dietary NAC can stimulate the growth of the small intestinal mucosa in response to endotoxin treatment. Interestingly, not all segments of the small intestine were affected by LPS in the same manner. In pigs, the duodenum has a different anatomical structure (e.g., villus height and crypt depth) and a different pattern of protein expression than other segments of the small intestine. This may explain why the duodenum of the LPS-treated pigs responded differently to NAC than their jejunum and ileum.

One of the putative mechanisms responsible for the action of NAC may involve expression of caspase-3. Caspase-3 is one of the key components of the apoptotic pathway in the small intestine (Tan et al. 2010). This protein is either partially or totally responsible for the proteolytic cleavage of many key proteins. Much evidence shows that LPS induces cell death through the activation of caspase-3. Of particular note, we found that NAC attenuated the production of active caspase-3 in the small intestine of LPS-challenged pigs (Fig. 1). This result indicates a protective effect of NAC against LPS-induced enterocyte death and supports the notion that dietary supplementation with NAC is effective in preventing intestinal oxidative injury and inflammatory disease in neonates. It should be noted, however, that NAC supplementation only partially ameliorated the LPS-induced changes in the measured intestinal variables. We surmise that LPS affects intestinal function through more than one pathway. Pathway(s) other than anti-oxidative reactions may be resistant to NAC. Alternatively, the dose of NAC used may not be sufficient to block all the adverse effects of LPS.

It is possible that NAC affects the expression of key proteins involved in anti-inflammatory responses via the tight junction signaling. Intestinal epithelial integrity was maintained by cohesive interactions between cells via the formation of tight junctions (Boudreau et al. 2007). Recent evidence suggests that members of the claudin family play a critical role in tight junction formation and determine permeability characteristics in a variety of tissues, including the gut (Howe et al. 2005; Tsukita and Furuse 2000). Claudin-1 and occludin integrate such diverse processes as gene transcription, tumor suppression and cell proliferation (Rhoads and Wu 2009; Schneeberger and Lynch 2004). LPS disrupts barrier function and increases paracellular permeability in a time- and dose-dependent manner and also induces a redistribution of tight junction proteins [occludin, claudin-1, claudin-4 and zonula occludens (ZO)-1] from the intercellular junctions and reduces the expression of ZO-1 (Sheth et al. 2007). To extend these observations, our results convincingly indicate that LPS reduced the abundance of claudin-1 and occludin in the small intestinal mucosa of piglets (Fig. 2, 3). It is noteworthy that dietary supplementation with NAC can increase the levels of claudin-1 and occludin in the small intestine of LPS-challenged piglets. Collectively, these data support the conclusion that NAC, a precursor of glutathione, can effectively protect the small intestine from oxidative injury and is a safe agent for clinical management of neonates with intestinal inflammation. Such an

effect of NAC is similar to that of arginine (Tan et al. 2010), although these two substances are not related in structure or metabolism. We have previously reported that arginine can reduce the expression of Toll-like receptor 4 (Tan et al. 2010) and increase the expression of glutathione-synthetic enzymes (Jobgen et al. 2009), which is expected to enhance anti-oxidative responses.

In conclusion, dietary supplementation with 500 mg/kg NAC alleviates intestinal injury and improves intestinal absorption in LPS-challenged piglets. The effects of NAC are associated with reduced cell death (indicated by decreased expression of the caspase-3 protein in the small intestinal mucosa of LPS-challenged piglets) and increased tight junctions (indicated by increased expression of claudin-1 and occludin proteins in the small intestinal mucosa of LPS-challenged piglets). Therefore, NAC beneficially relieves the growth depression induced by endotoxin. These findings not only aid in understanding the mode of NAC's actions in the gut of piglets, but also have important implications for improving infant nutrition and management under inflammatory conditions.

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

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