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ORIGINAL ARTICLE

N-acetylcysteine reduces inflammation in the small intestine by regulating redox, EGF and TLR4 signaling

Yongqing Hou · Lei Wang · Dan Yi · Binying Ding · Zhenguo Yang · Jiao Li · Xing Chen · Yinsheng Qiu · Guoyao Wu

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Abstract This study determined whether *N*-acetylcysteine (NAC) could affect intestinal redox status, proinflammatory cytokines, epidermal growth factor (EGF), EGF receptor (EGFR), Toll-like receptor-4 (TLR4), and aquaporin-8 in a lipopolysaccharide (LPS)-challenged piglet model. Eighteen piglets (35-day-old) were randomly allocated into one of the three treatments (control, LPS and NAC). The control and LPS groups were fed a basal diet, and the NAC group received the basal diet +500 mg/kg NAC. On days 10, 13, and 20 of the trial, the LPS- and NAC-treated piglets received intraperitoneal administration of LPS (100 μg/kg BW), whereas the control group received the same volume of saline. On days 10 and 20, venous blood samples were obtained at 3 h post LPS or saline injection. On day 21 of the trial, piglets were killed to obtain the intestinal mucosa for analysis. Compared with the control group, LPS challenge reduced ($P < 0.05$) the activities of superoxide dismutase, catalase, and glutathione peroxidase in jejunal mucosae, while increasing ($P < 0.05$) the concentrations of malondialdehyde, H_2O_2, $O_2^{\cdot -}$ and the ratio of oxidized to reduced glutathione in jejunal mucosae, and concentrations of TNF-α, cortisol, interleukin-6, and prostaglandin E_2 in both plasma and intestinal mucosae. These adverse effects of LPS were attenuated ($P < 0.05$) by NAC supplementation. Moreover, NAC prevented LPS-induced increases in abundances of intestinal HSP70 and NF-κB p65 proteins and TLR4 mRNA. NAC supplementation enhanced plasma EGF concentration and intestinal EGFR mRNA levels. Collectively, these results indicate that dietary NAC supplementation alleviates LPS-induced intestinal inflammation via regulating redox, EGF, and TLR4 signaling.

Keywords *N*-acetylcysteine · Intestinal mucosa · Lipopolysaccharide · Epidermal growth factor · Toll like receptor · Aquaporin

Abbreviations

AQP	Aquaporin
EGF	Epidermal growth factor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HSP70	Heat shock protein 70
LPS	Lipopolysaccharide
NF-κB	Nuclear factor κB
RT-PCR	Real-time polymerase-chain reaction
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-alpha

Introduction

N-acetylcysteine (NAC) is a precursor of L-cysteine and reduced glutathione (Wu et al. 2004). When interacting with reactive oxygen species (ROS), NAC acts as a scavenger of free radicals (Zafarullah et al. 2003). NAC is a source of sulfhydryl groups in cells and these sulfur-containing substances play critical roles in the body, including

Y. Hou (✉) · L. Wang · D. Yi · B. Ding · Z. Yang · J. Li · X. Chen · Y. Qiu
Hubei Key Laboratory of Animal Nutrition and Feed Science, Wuhan Polytechnic University, 430023 Wuhan, China
e-mail: houyq777@yahoo.com.cn

G. Wu
State Key Laboratory of Animal Nutrition, China Agricultural University, 100193 Beijing, China

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

detoxification and protecting cells and cellular components against oxidative stress (Wu et al. 2004). Thus, NAC has beneficial effects on detoxification and preventing diseases, including cancer, heart disorders, human immunodeficiency virus infection, acetaminophen-induced liver toxicity, and metal toxicity (Yu 1994). NAC is rapidly metabolized by the small intestine to produce glutathione (Hou et al. 2011c) and it cannot be detected in animals without supplementation (Wu 2009). We recently demonstrated that dietary supplementation with 500 mg/kg NAC alleviated the mucosal damage and improved the absorptive function of the small intestine in LPS-challenged piglets (Hou et al. 2011c), but the underlying mechanisms are largely unknown.

It is generally thought that the physiological function of NAC is attributed to its antioxidative action via the synthesis of L-cysteine and GSH (Zafarullah et al. 2003), but other possible effects of NAC (e.g., regulation of cell signaling independent GSH) have not been reported for any species. Available evidence shows that LPS induces intestinal mucosal oxidative stress in animals, including pigs (Hou et al. 2011b). However, it remains unknown whether dietary supplementation with NAC can alleviate the adverse effects of LPS on intestinal inflammation and damage. Expression of epithelial growth factor (EGF) and the EGF receptor (EGFR) were reported to positively correlate with the recovery of the small intestinal mucosa (Helmrath et al. 1998; Nair et al. 2008). Aquaporins (AQP; water-transporting proteins), which constitute a family of small integral membrane proteins that are selectively permeable to water, are driven by osmotic gradients (Takata et al. 2004). A reduction in AQP expression appears to be associated with ulcerative colitis and Crohn's colitis (Hardin et al. 2004). AQP8 has been proposed to be a potentially important water transporter in the gastrointestinal tract (Yamamoto et al. 2007). Some AQP isoforms (AQP1, AQP5, and AQP8) were downregulated by LPS in vitro or in adult rats (Lehmann et al. 2008; Yao et al. 2010), but no studies had been conducted with neonates or other species. AQP8 is the major water transporter in the gastrointestinal tract and is present in the absorptive epithelial cells of the duodenum, jejunum, and colon, whereas AQP1 and AQP5 are expressed mainly in other tissues (Matsuzaki et al. 2004). At present, it is unknown whether NAC can prevent the LPS-induced downregulation of intestinal AQP expression. In addition to AQP, TLR4 signaling is also associated with intestinal inflammation (Gribar et al. 2008; Kawai and Akira 2006). However, it is unknown whether there is a link between NAC and TLR4 signaling under conditions of intestinal inflammation.

Based on the foregoing, we hypothesized that NAC may ameliorate LPS-induced intestinal inflammation by regulating EGF, TLR4, and AQP8 expression. The piglet, a well-established animal model for studying infant nutrition and gastrointestinal disease (Geng et al. 2011; Li et al. 2009; Wilson et al. 2011), was used to test this hypothesis. The current work was derived from our published study (Hou et al. 2011c), but the data reported herein have not previously been published elsewhere.

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. Eighteen crossbred healthy female piglets (Duroc × Landrace × Yorkshire) were reared by sows and then weaned at 28 ± 2 days of age. After 7-day adaptation, the piglets (35 ± 2 days of age, average body weight of 11.6 ± 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, c; Tan et al. 2009a). The maize- and soybean meal-based diet was formulated to meet National Research Council (1998)-recommended requirements for all nutrients (Hou et al. 2011c).

Experimental design

All piglets had free access to the basal diet between days 28 and 35 of age (days 0–7 postweaning) for adapting to solid food (Hou et al. 2011c). At 35 days of age, piglets were assigned randomly into one of the three treatment groups: (1) control group (non-challenged control, piglets fed the basal diet and receiving intraperitoneal administration of sterile saline); (2) LPS group (LPS-challenged control, piglets fed the basal diet and receiving intraperitoneal administration of *Escherichia coli* LPS); and (3) NAC group (LPS + 500 mg/kg NAC, piglets fed the basal diet supplemented with 500 mg/kg NAC and receiving intraperitoneal administration of *Escherichia coli* LPS). There were six piglets per group. LPS was dissolved in sterile saline. NAC (powder) was well mixed with the basal diet. The diets of the control and LPS groups were supplemented with 500 mg/kg cornstarch 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 alleviate the mucosal damage and improves the absorptive function of the small intestine in LPS-challenged piglets (Hou et al. 2011c). 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 an isonitrogenous control. On days 10, 13, and 20 of the trial, overnight fasted piglets in the LPS group and NAC group 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 sterile saline. During days 0–10 of the trial (pre-challenge), all the piglets had free access to food and drinking water. To exclude a possible effect of LPS-induced reduction in food intake on the piglet intestine, the control and 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. At 24 h post-injection of LPS or saline (on day 21), all piglets were killed under anesthesia with an intravenous injection of pentobarbital sodium (50 mg/kg BW) for the biochemical analysis of the intestinal mucosa (Hou et al. 2010; Deng et al. 2009).

Collection of blood samples

On days 10 and 20 of the trial, 3 hours after LPS or saline administration, blood samples (10 mL) were collected from the anterior vena cava into heparinized vacuum tubes (Becton–Dickinson Vacutainer System, Franklin Lake, NJ, USA), as previously described (Hou et al. 2011a, c). Blood samples (7 mL) 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.

Collection of intestinal mucosal samples

The piglet abdomen was surgically 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 10-cm segments were cut at distal duodenum, mid-jejunum, and mid-ileum (Hou et al. 2010; Wang et al. 2008). The intestinal segments were opened longitudinally and the contents were flushed gently with ice-cold phosphate buffered saline (pH 7.4) (Hou et al. 2010). 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 15 min after killing.

Concentrations of anti-oxidative enzymes and related products in jejunal mucosa

Jejunal mucosae were used for the analysis of anti-oxidative enzymes and related products. Specifically, superoxide radical was determined as described by Hou et al. (2011a). Reduced and oxidized glutatione were analyzed using commercially available kit (Beyotime Institute of Biotechnology, Jiangsu, China). Activities of superoxide dismutase, catalase, and glutathione peroxidase, as well as malondialdehyde and hydrogen peroxide were determined using commercially available kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Assays were performed in triplicate.

Determination of proinflammatory cytokines in plasma and intestinal mucosae

Frozen intestinal mucosal samples were powdered under liquid nitrogen and then homogenized in ice-cold 0.9 % NaCl solution with a homogenizer. The weight of sample: the volume of 0.9 % NaCl = 1:9. The homogenates were centrifuged at 3,000 rpm for 15 min at 4 °C to obtain the supernatant fluid.

Tumor necrosis factor-α (TNF-α) and cortisol in plasma and the supernatant fluid of intestinal mucosae were analyzed using commercially available ^{125}I RIA kits (Beijing North Institute of Biological Technology, Beijing, China). The detection limit was 0.03 ng/mL for TNF-α and 0.2 ng/mL for cortisol. The intra-and inter-assay coefficients of variation were 5 and 8 % for TNF-α, respectively, and were <10 and <15 % for cortisol, respectively.

Interleukin-6 and prostaglandin E_2 in plasma and the supernatant fluid of intestinal mucosae were analyzed using commercially available ^{125}I RIA kits (Beijing Sino-UK Institute of Biological Technology, Beijing, China). The detection limits for interleukin-6 and prostaglandin E_2 analyses were 5.0 and 0.12 pg/mL, respectively. The coefficients of variation for intra-and inter-assays of interleukin-6 were <7 and <15 %, respectively. The coefficients of variation for intra- and inter-assays of prostaglandin E_2 were <7.5 and <10.5 %, respectively.

Determination of EGF in plasma

EGF in plasma was analyzed using a commercially available ^{125}I RIA kit (Beijing Sino-UK Institute of Biological Technology). Human EGF antibody was used as the standard. The intra-and inter-assay coefficients of variation were <5 and <10 %, respectively. The detection limit was 0.1 μg/L.

Determination of EGFR, TLR4, and AQP 8 mRNA levels using real-time polymerase-chain reaction

EGFR, TLR4, and AQP 8 mRNA levels in jejunal and ileal mucosae were quantified using RT-PCR. Approximately 100 mg of a frozen mucosal sample was powdered under liquid nitrogen using a mortar and pestle. The powdered

samples were homogenized and total RNA was isolated using the TRIzol Reagent protocol (Invitrogen, Carlsbad, CA, USA). Total RNA was quantified using the NanoDrop® ND-2000 UV–VIS spectrophotometer (Thermo Scientific, Wilmington, DE, USA) at an OD of 260 nm, and the purity was assessed by determining the OD260/OD280 ratio. All of the samples had an OD260/OD280 ratio above 1.8 corresponding to 90–100 % pure nucleic acids. Meanwhile, the integrity of RNA in each sample was assessed using 1 % denatured agarose gel electrophoresis. RNA was used for RT-PCR analysis when it had a 28 S/18 S rRNA ratio ≥1.8.

Total RNA was reverse-transcribed using the PrimeScript® RT reagent Kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's instruction. cDNA was synthesized and stored at −20 °C until use. The RT-PCR analysis of gene expression was performed using primers for EGFR, TLR4, AQP 8, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Table 1) and the SYBR® Premix Ex Taq™ (Takara, Dalian, China) on an Applied Biosystems 7500 Fast Real-Time PCR System (Foster City, CA, USA). The total volume of PCR reaction system was 50 μL. In brief, the reaction mixture contained 0.2 μM of each primer, 25 μL of SYBR® Premix Ex Taq™ (2×), and 4 μL of cDNA in a 50-μL reaction volume. All PCRs were done in triplicate on a 96-well RT-PCR plate (Applied Biosystems) under the following conditions (two-step amplification): 95 °C for 30 s, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. A subsequent melting curve (95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s) with continuous fluorescence measurement and final cooling to room temperature was processed. Amplification products were verified by melting curves, agarose gel electrophoresis, and direct sequencing. Results were analyzed by the cycle threshold (C_T) method (Fu et al. 2010). Each biological sample was run in triplicate.

Table 1 Primers for RT-PCR analysis

Gene	Primers	
EGFR	Forward	5′-GGCCTCCATGCTTTTGAGAA -3′
	Reverse	5′-GACGCTATGTCCAGGCCAA-3′
TLR4	Forward	5′-GCCTTTCTCTCCTGCCTGAG -3′
	Reverse	5′-AGCTCCATGCATTGGTAACTAATG-3′
AQP8	Forward	5′-TGTGTCTGGAGCCTGCATGAAT-3′
	Reverse	5′-AGCAGGAATCCCACCATCTCA-3′
GAPDH	Forward	5′-CGTCCCTGAGACACGATGGT-3′
	Reverse	5′-CCCGATGCGGCCAAAT-3′

The oligonucleotide primers were designed from pig gene sequences in the GenBank NM-2140075 (for EGFR), AB188301 (for TLR4), NM_001112683 (for AQP 8), and AF017079 (for GAPDH). To avoid amplification of potentially contaminating genomic DNA, the primers were designed to span introns and intron–exon boundaries

Protein immunoblot analysis

HSP70 and NF-κB p65 proteins were analyzed by western blotting as described by Hou et al. (2010, 2011a, c). 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 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 bromphenol 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 (50 μg/sample for HSP70; 60 μg/sample for NF-κB p65) were separated by electrophoresis on a 10 % polyacrylamide gel. Proteins were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. Skim-milk powder in TBS/T buffer was used to block membranes for at least 1 h at room temperature (Yao et al. 2011). Membranes were incubated with primary antibodies overnight at 4 °C: HSP70 (mouse monoclonal antibody from Stressgen Bioreagents, Columbia, Canada, dilution 1:2,000 in primary antibody dilution buffer), NF-κB p65 (mouse polyclonal antibodies from Cell Signaling, Danvers, MA, USA; dilution 1:1,000 in primary antibody dilution buffer) or β-actin (mouse monoclonal antibody from Sigma Chemicals; dilution 1:5,000 in primary antibody dilution buffer). The primary antibody dilution buffer was 1 × TBS, 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-goat (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, and quantified using an imaging system (Alpha Innotech FluorChem FC2, CA, USA).

Statistical analysis

Data, expressed as mean values ± SEM, were analyzed by one-way analysis of variance. The normality and constant variance for experimental data were tested by the Levene's test (Wei et al. 2012). If data did not have homogenous

variance, they underwent logarithm transformation to meet the necessary assumptions of analysis of variance (Wei et al. 2012). Differences among treatment means were determined by Duncan's multiple range tests. All statistical analyses were performed using SPSS 13.0 software (Chicago, IL, USA). P values <0.05 were taken to indicate statistical significance, and P values <0.1 were considered as trends toward statistical significance.

Results

Effects of NAC on jejunal redox status

Data on concentrations of antioxidative enzymes and related products in jejunal mucosae are summarized in Table 2. Compared with the control group, LPS piglets exhibited decreases ($P < 0.05$) in the activities of superoxide dismutase, catalase, and glutathione peroxidase in the jejunal mucosa, as well as increases ($P < 0.05$) in the concentrations of malondialdehyde, hydrogen peroxide, and superoxide radical and the ratio of oxidized to reduced glutathione. In comparison with the LPS piglets, NAC supplementation increased ($P < 0.05$) the concentrations of superoxide dismutase and catalase in the jejunal mucosa, while reducing ($P < 0.05$) the concentrations of malondialdehyde, hydrogen peroxide, and superoxide radical and the ratio of oxidized to reduced glutathione in the jejunal mucosa (Table 2).

Concentrations of inflammatory mediators in plasma and intestinal mucosae

Data on concentrations of inflammatory mediators in plasma and intestinal mucosae are summarized in Table 3. Compared with the control group, LPS piglets had higher ($P < 0.05$) concentrations of TNF-α, cortisol, interleukin 6, and prostaglandin E_2 in plasma (on days 10 and 20 of the trial), and intestinal mucosae. These effects of LPS were ameliorated ($P < 0.05$) by dietary supplementation with NAC.

Abundance of HSP70 protein in intestinal mucosae

The small intestine of young pigs expressed HSP70 (Fig. 1). Compared with the control group, LPS administration resulted in an increase ($P < 0.05$) in HSP70 expression in duodenal, jejunal, and ileal mucosae. Notably, in comparison with LPS piglets, dietary supplementation with NAC decreased ($P < 0.05$) the abundance of HSP70 protein in duodenal, jejunal, and ileal mucosae (Fig. 1).

Abundance of NF-κB p65 protein in intestinal mucosa

NF-κB p65 was readily detected in the piglet small intestine (Fig. 2). LPS administration resulted in an increase ($P < 0.05$) in abundance of the NF-κB p65 protein in jejunal and ileal mucosae, compared with control piglets. Relative to the LPS group, dietary supplementation with 500 mg/kg NAC decreased ($P < 0.05$) NF-κB p65 expression in the jejunum and ileum (Fig. 2).

Concentrations of EGF in plasma

Data on concentrations of EGF in plasma are shown in Table 3. Compared with control piglets, LPS challenge resulted in a decrease ($P < 0.05$) in concentrations of EGF in plasma on days 10 and 20 of the trial (after the first and last LPS challenges). Relative to LPS piglets, dietary supplementation with NAC increased ($P < 0.05$) the concentrations of EGF in plasma.

Table 2 Effects of NAC on activities of antioxidative enzymes and redox status in the jejunal mucosa

Items	Control group	LPS group	NAC group
SOD (U/mg protein)	85.5 ± 2.5^{a}	71.8 ± 1.8^{b}	78.5 ± 2.2^{a}
CAT (U/g protein)	52.4 ± 3.1^{a}	27.5 ± 0.70^{c}	37.3 ± 2.7^{b}
GSH-Px (U/g protein)	85.4 ± 6.8^{a}	57.1 ± 7.2^{b}	70.7 ± 4.4^{ab}
MDA (μmol/g protein)	0.37 ± 0.02^{b}	0.48 ± 0.01^{a}	0.36 ± 0.05^{b}
H_2O_2 (μmol/g protein)	0.66 ± 0.01^{b}	0.77 ± 0.03^{a}	0.68 ± 0.03^{b}
$O_2^{\cdot-}$ (μmol/g protein)	2.52 ± 0.08^{c}	4.76 ± 0.43^{a}	3.22 ± 0.10^{b}
GSSG/GSH	0.10 ± 0.01^{b}	0.23 ± 0.03^{a}	0.11 ± 0.01^{b}

Data are mean values ± SEM, $n = 6$. Values within a row with different letters differ ($P < 0.05$)

Control group (non-challenged control) piglets fed the basal diet and injected with saline, *LPS group* (LPS challenged control) piglets fed the basal diet and challenged with *Escherichia coli* LPS, *NAC group* (LPS + 500 mg/kg NAC) piglets fed the basal diet supplemented with 500 mg/kg and challenged with LPS, *CAT* catalase, *GSSG* oxidized glutathione, *GSH* reduced glutathione, *GSH-Px* glutathione peroxidase, H_2O_2 hydrogen peroxide, *MDA* malondialdehyde, $O_2^{\cdot-}$ superoxide anion, *SOD* superoxide dismutase

Table 3 Effects of NAC on concentrations of proinflammatory mediators in plasma and intestinal mucosae and of EGF in plasma

Item	Control group	LPS group	NAC group
Plasma (day 10)			
TNF-α (ng/mL)	0.68 ± 0.19^{c}	3.48 ± 0.34^{a}	2.25 ± 0.24^{b}
Cortisol(ng/mL)	22.0 ± 6.4^{c}	272 ± 29^{a}	201 ± 8^{b}
IL-6 (pg/mL)	109 ± 6.4^{b}	210 ± 19^{a}	136 ± 19^{b}
PGE_2 (pg/mL)	51.1 ± 3.3^{c}	71.1 ± 2.9^{a}	62.3 ± 1.9^{b}
EGF (ng/mL)	1.03 ± 0.04^{a}	0.83 ± 0.05^{b}	1.05 ± 0.08^{a}
Plasma (day 20)			
TNF-α (ng/mL)	0.63 ± 0.01^{b}	1.14 ± 0.11^{a}	0.66 ± 0.14^{b}
Cortisol (ng/mL)	22.0 ± 5.4^{b}	117 ± 23^{a}	66.5 ± 14.0^{ab}
IL-6 (pg/mL)	104 ± 9^{b}	156 ± 4^{a}	119 ± 19^{b}
PGE_2 (pg/mL)	50.3 ± 1.1^{b}	60.6 ± 2.6^{a}	52.4 ± 3.0^{b}
EGF (ng/mL)	1.26 ± 0.11^{a}	0.82 ± 0.09^{b}	1.35 ± 0.17^{a}
Duodenal mucosa			
TNF-α (ng/mL)	2.19 ± 0.34^{b}	4.46 ± 0.51^{a}	3.18 ± 0.30^{b}
Cortisol (ng/mL)	2.30 ± 0.11^{b}	5.99 ± 0.76^{a}	4.77 ± 0.69^{a}
IL-6 (pg/mL)	174 ± 8^{b}	267 ± 22^{a}	206 ± 37^{ab}
PGE_2 (pg/mL)	81.7 ± 5.4	91.8 ± 2.7	84.5 ± 3.1
Jejunal mucosa			
TNF-α (ng/mL)	2.92 ± 0.45^{b}	5.11 ± 0.18^{a}	3.16 ± 0.28^{b}
Cortisol (ng/mL)	2.29 ± 0.23^{b}	5.45 ± 0.87^{a}	3.49 ± 0.49^{b}
IL-6 (pg/mL)	210 ± 19^{b}	366 ± 18^{a}	263 ± 20^{b}
PGE_2 (pg/mL)	80.1 ± 1.6^{b}	95.7 ± 4.5^{a}	82.4 ± 2.2^{b}
Ileal mucosa			
TNF-α (ng/mL)	1.61 ± 0.26^{b}	3.47 ± 0.44^{a}	2.18 ± 0.32^{b}
Cortisol (ng/mL)	2.73 ± 0.52^{c}	7.87 ± 0.88^{a}	4.81 ± 0.52^{b}
IL-6 (pg/mL)	142 ± 15^{b}	235 ± 23^{a}	144 ± 7^{b}
PGE_2 (pg/mL)	86.0 ± 3.1^{b}	104 ± 3^{a}	92.9 ± 2.5^{b}

Data are mean values ± SEM, $n = 6$. Values within a row with different letters differ ($P < 0.05$)

Control group (non-challenged control) piglets fed the basal diet and injected with saline, *LPS group* (LPS challenged control) piglets fed the basal diet and challenged with *Escherichia coli* LPS, *NAC group* (LPS + 500 mg/kg NAC) piglets fed the basal diet supplemented with 500 mg/kg and challenged with LPS, *IL-6* interleukin 6, *PGE_2* prostaglandin E_2

EGFR, TLR4, and AQP8 mRNA levels in jejunal and ileal mucosae

The jejunal and ileal mucosae of young pigs expressed the genes for EGFR, TLR4, and AQP8 (Table 4). Mucosal EGFR mRNA levels were lower ($P < 0.05$) in the jejunum (−36 %) and ileum (−28 %) of LPS-treated piglets than in the control group. Compared with the LPS group, NAC supplementation increased the abundance of EGFR mRNA in jejunal and ileal mucosae by 19 % ($P < 0.10$) and 38 % ($P < 0.05$), respectively. In contrast to the EGFR gene expression, mucosal TLR4 mRNA levels were higher ($P < 0.05$) in the jejunum (+107 %) and ileum (+89 %) of LPS-treated piglets than in the control group. Compared with the LPS piglets, NAC supplementation decreased TLR4 mRNA abundance in jejunal and ileal mucosae by 47 % ($P < 0.05$) and 27 % ($P < 0.1$), respectively. Jejunal mucosal AQP mRNA levels were lower ($P < 0.05$) in the LPS-treated piglets than in the control group. No difference in AQP mRNA levels were detected ($P > 0.10$) in the ileal mucosa among the three groups of piglets. NAC supplementation did not affect ($P > 0.10$) the abundance of AQP mRNA in the jejunal or ileal mucosa.

Discussion

The intestinal integrity plays an essential role in nutrition (Bergen and Wu 2009; Dai et al. 2012), metabolism (Brosnan and Brosnan 2012; Rezaei et al. 2011), and

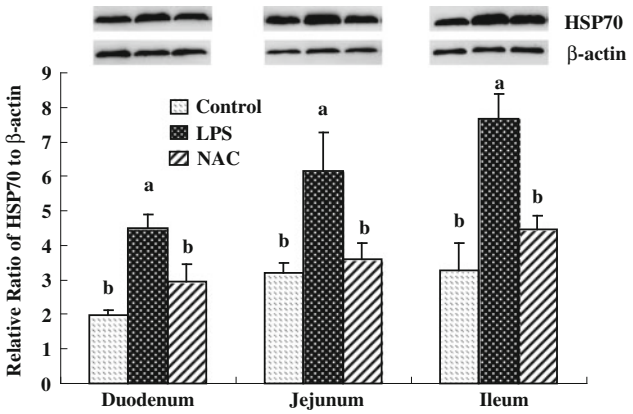

Fig. 1 Relative levels of heat shock protein 70 (HSP70) expressed in small intestinal mucosa of piglets. Mucosal extracts (50 μg protein/sample) were separated by 10 % SDS–polyacrylamide gel electrophoresis for determination of HSP70 and *β*-actin. Values for relative HSP70 were normalized for *β*-actin. Data are mean values ± SEM, $n = 6$. *Control* (non-challenged control) piglets fed the basal diet and injected with sterile saline; *LPS* (LPS challenged control) piglets fed the same control diet and challenged with *Escherichia coli* LPS; *NAC* (LPS + 500 mg/kg NAC) piglets fed the basal diet supplemented with 500 mg/kg NAC and challenged with LPS. *a*, *b* Within same intestinal segment, means with different superscripts differ ($P < 0.05$)

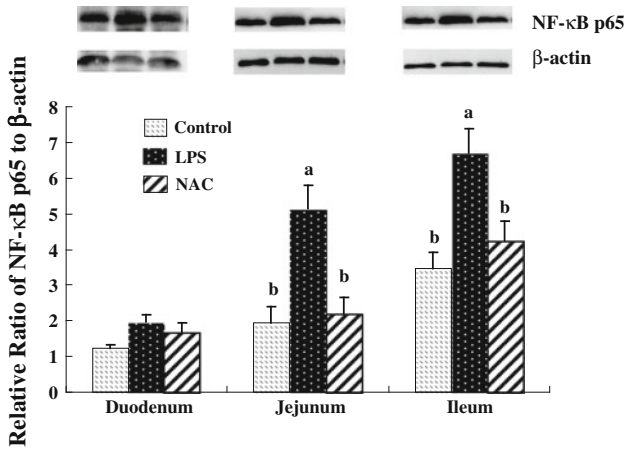

Fig. 2 Relative levels of nuclear factor κB (NF-κB p65) expressed in small intestinal mucosa of piglets. Mucosal extracts (60 μg protein/sample) were separated by 10 % SDS–polyacrylamide gel electrophoresis for determination of NF-κB p65 and *β*-actin. Values for relative NF-κB p65 were normalized for *β*-actin. Data are mean values ± SEM, $n = 6$. *Control* (non-challenged control) piglets fed the bsasl diet and injected with sterile saline; *LPS* (LPS challenged control) piglets fed the same control diet and challenged with *Escherichia coli* LPS; *NAC* (LPS + 500 mg/kg NAC) piglets fed the basal diet supplemented with 500 mg/kg NAC and challenged with LPS. *a*, *b* Within same intestinal segment, means with different superscripts differ ($P < 0.05$)

whole-body homeostasis (Dai et al. 2011; Xi et al. 2011). To evaluate whether NAC supplementation could ameliorate the adverse effects of intestinal injury, we used a well-established animal model of LPS-induced inflammation in piglets (Hou et al. 2010, 2011a, b, c; Liu et al. 2008; Mercer et al. 1996). Gut mucosal damage occurs in response to LPS administration (Lobo et al. 2003), which is a potent endotoxin that can be absorbed readily into the systemic circulation via the gastrointestinal tract (Liu et al. 2008). In addition to the possible effects of hypoperfusion on endotoxin-induced gut injury, LPS reduces oxidative metabolism in the gastrointestinal tract (Lobo et al. 2003). This is consistent with our finding that LPS challenge caused intestinal oxidative stress (Table 2). The effects of LPS are due, in part, to its ability to stimulate macrophages to synthesize and secrete proinflammatory cytokines (Lehmann et al. 2008). Thus, LPS enhances the production of inflammatory cytokines by multiple organs, including the small intestine (Webel et al. 1997). In keeping with this notion, we found that LPS administration increased the concentrations of TNF-α, interleukin-6, cortisol, and prostaglandin E_2 in plasma and intestinal mucosae (Table 3).

Reactive oxygen species, such as superoxide radical, hydrogen peroxide, and hydroxyl radical, are produced primarily by the mitochondria in cells as a by-product of normal metabolism during conversion of molecular oxygen to water (Fang et al. 2002). However, cells possess defense mechanisms against free radicals and other oxidants. The anti-oxidative enzymes include superoxide dismutase, catalase, and glutathione peroxidase (Yu 1994). Superoxide dismutase converts superoxide anion ($O_2^{\cdot-}$) into H_2O_2 and O_2 (Zafarullah et al. 2003). H_2O_2 is degraded to water by catalase. Glutathione peroxidase protects intracellular organelles from the damaging effects of hydroperoxides. This enzyme catalyses the reduction of H_2O_2 to water, with the simultaneous conversion of reduced glutathione to oxidized glutathione (Wu et al. 2004). The circulating level of malondialdehyde, which is a reactive oxygen species, serves as a useful bio-marker of in vivo oxidative stress (Fang et al. 2002).

A new and important finding of the present study is that the adverse effects of LPS on intestinal oxidative stress were attenuated by dietary NAC supplementation (Tables 2, 3). As a scavenger of free radicals, NAC interacts with oxidants (Zafarullah et al. 2003) and protects cells against oxidative damage (Knight et al. 1991). The antioxidant effects of NAC may be directly related to its chemical structure or to the secondary induction of glutathione production. Among direct effects of NAC are reactions with hydroxyl radicals, resulting in their inactivation (Fishbane et al. 2004). In this process, NAC is converted into NAC thiol radical intermediates and finally into NAC disulfide (Zafarullah et al. 2003). A secondary antioxidant effect of NAC is exerted indirectly via the increase in glutathione synthesis, which is essential for cellular defense against oxidative damage (Fishbane et al. 2004). In support of this view, treatment with NAC at 1 h before endotoxin administration attenuated NF-κB

Table 4 Effects of NAC on EGFR, TLR4, and AQP8 mRNA levels in jejunal and ileal mucosae

Items	Control group	LPS group	NAC group
EGFR mRNA			
Jejunum	1.00 ± 0.06^{a}	0.64 ± 0.07^{b}	0.76 ± 0.08^{ab}
Ileum	1.00 ± 0.05^{a}	0.72 ± 0.05^{b}	0.99 ± 0.07^{a}
TLR4 mRNA			
Jejunum	1.00 ± 0.24^{b}	2.07 ± 0.42^{a}	1.09 ± 0.16^{b}
Ileum	1.00 ± 0.19^{b}	1.89 ± 0.28^{a}	1.38 ± 0.17^{ab}
AQP8 mRNA			
Jejunum	1.00 ± 0.08^{a}	0.42 ± 0.05^{b}	0.52 ± 0.09^{b}
Ileum	1.00 ± 0.16	1.11 ± 0.32	1.13 ± 0.45

Data are means ± SEM, $n = 6$. Values within a row with different letters differ ($P < 0.05$)

Control group (non-challenged control) piglets fed the basal diet and injected with saline, *LPS group* (LPS challenged control) piglets fed the basal diet and challenged with *Escherichia coli* LPS, *NAC group* (LPS + 500 mg/kg NAC) piglets fed the basal diet supplemented with 500 mg/kg and challenged with LPS

activation in the lung tissue of rats (Blackwell et al. 1996). Through suppressing NF-κB activation, NAC also inhibited the production of nitric oxide by the inducible form of nitric oxide synthetase and the generation of interleukin-6 by cells of the immune system (Li et al. 2007). In line with these reports, our results indicated that dietary supplementation with NAC prevented the LPS-induced increase in NF-κB expression in the small-intestinal mucosa (Fig. 2). NF-κB is normally bound to IκB protein in the cytoplasm, but is released to enter the nucleus in response to infection, oxidative stress, or pro-inflammatory cytokines (Kawai and Akira 2006). The release of NF-κB can increase transcription of genes coding for TNF-α, interleukin-1, and interleukin-6, which ultimately result in a positive feedback loop. TNF-α causes ubiquitination and subsequent degradation of IκB by proteases. It is noteworthy that NAC blocks TNF-α activation of NF-κB independently of its antioxidant activity by causing structural changes in the TNF receptor that lower receptor affinity for TNF-α (Lehmann et al. 2008).

The 70-kDa HSP-70 belongs to a family of ubiquitously expressed heat shock proteins (Kregel 2002). HSP70 proteins protect cells from thermal or oxidative stress (Rhoads and Wu 2009). These stresses normally act to damage proteins, causing their partial unfolding and possible aggregation. By temporarily binding to hydrophobic residues, HSP70 prevents these partially denatured proteins from aggregating and allows them to refold (Kregel 2002). In addition to improving overall protein integrity, HSP 70 directly inhibits apoptosis (Beere et al. 2000). Additionally, a high concentration of HSP70 is indicative of oxidative stress (Hou et al. 2010). In our study, higher HSP70 expression in intestinal mucosa in the LPS group than in the NAC group indicated that supplementation of NAC substantially decreased oxidative stress in the intestinal mucosa of piglets (Fig. 1).

Another significant finding of the present study is that dietary supplementation with NAC increased EGF concentration in plasma and expression of the EGFR gene in jejunal and ileal mucosae (Table 4). Previous studies have demonstrated that EGF and EGFR were related to the damage and recovery of the small intestinal mucosa (Helmrath et al. 1998; Nair et al. 2008). EGF and EGFR are crucial for stimulating enterocyte proliferation and regeneration of the mucosal epithelium (Engler et al. 1999; Ryan et al. 1997; Thomson et al. 2001). EGF may not have an effect on the intestinal mucosal epithelium in healthy animals, can promote cell proliferation, repair, and migration during regeneration following damage (Helmrath et al. 1998; Nair et al. 2008). The signal transduction mediated by EGFR is characterized by a plethora of beneficial responses, including the enhancement of cell proliferation, repair and migration, and the stabilization of internal environment (Helmrath et al. 1998). Similarly, we found that 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) (Hou et al. 2011c). Therefore, it is possible that NAC may alleviate intestinal injury partly via the EGF signaling.

Toll-like receptors are type I transmembrane receptors that are expressed on the cell membrane after LPS stimulation (Kawai and Akira 2006). Previous studies have demonstrated that TLR4 activation leads to an increase in enterocyte apoptosis (Tan et al. 2010) and a loss of barrier integrity (Leaphart et al. 2007). In addition to activating pathways leading to cytokine release, TLR4 may directly contribute to perturbations in mucosal healing and further exacerbate the inflammatory response within the intestinal mucosa (Gribar et al. 2008). Consistent with these reports, our results indicated that LPS enhanced TLR4 expression in jejunal and ileal mucosae (Table 4). Notably, dietary supplementation with NAC reduced the elevated level of the TLR4 mRNA. Taken together with data on NF-κB and TNF-α expression (Table 4), these results provide evidence that NAC prevented the activation of the TLR4 signaling, alleviated inflammatory response and the mucosal damage in the small intestine. This work adds to the knowledge of functional amino acids in nutrition and health (e.g., Gao et al. 2011; Li et al. 2011a, b; Ren et al. 2011a, b; Satterfield et al. 2011, 2012; Wang et al. 2012; Wu 2010a, b; Wu et al. 2011a, b).

The results of this study also indicated that expression of the AQP8 gene was decreased in the jejunal mucosa of LPS piglets (Table 4). Patients with active ulcerative colitis, Crohn's colitis or infectious colitis who develop severe

diarrhea have similar reductions in intestinal AQP8 expression (Hardin et al. 2004; Yamamoto et al. 2007). Likewise, AQP4 and AQP8 mRNA levels were reduced in the proximal colon of allergic mice with severe diarrhea (Yamamoto et al. 2007). Dietary supplementation with NAC did not affect AQP8 mRNA levels in the intestinal mucosa of LPS-challenge piglets. Therefore, it may be possible that the effects of NAC on intestinal mucosa are independent of the transcription of the AQP8 gene. Future studies are warranted to determine the abundance and function of the AQP8 protein in the small-intestinal mucosa of NAC-supplemented animals.

In conclusion, dietary supplementation with 500 mg/kg NAC alleviates intestinal injury in LPS-challenged piglets. The beneficial effects of NAC are associated with the following changes in the small-intestinal mucosae: (1) reduced oxidative stress (indicated by increased activities of antioxidant enzymes, decreased production of reactive oxygen species, the lowered ratio of oxidized to reduced glutathione, and reduced expression of the HSP70 protein), (2) reduced inflammation (indicated by reduced concentrations of inflammatory mediators) via the TLR4 signaling (indicated by decreased expression of the NF-κB p65 protein and TLR4 mRNA), and (3) improved mucosal repair via the EGF signaling (indicated by increases in EGF production and EGFR expression). These novel findings have important implications for development of new interventions to ameliorate gut injury and dysfunction in animals and humans. Further research is warranted to understand how NAC affects the EGF and TLR4 signaling in the intestine.

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

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