Amino Acids (2009) 37:209–218
DOI 10.1007/s00726-009-0242-y

ORIGINAL ARTICLE

Proteomic analysis reveals altered expression of proteins related to glutathione metabolism and apoptosis in the small intestine of zinc oxide-supplemented piglets

Xiaoqiu Wang · Deyuan Ou · Jingdong Yin · Guoyao Wu · Junjun Wang

Received: 5 January 2009 / Accepted: 12 January 2009 / Published online: 29 January 2009
© Springer-Verlag 2009

Abstract Zinc is an important dietary factor that regulates intestinal amino acid and protein metabolism in animals. Recent work with the piglet, an established animal model for studying human infant nutrition, has shown that supplementing high levels of zinc oxide (ZnO) to the diet ameliorates weaning-associated intestinal injury and growth retardation. However, the underlying mechanisms are largely unknown. This study tested the hypothesis that zinc supplementation affects expression of proteins related to glutathione metabolism and oxidative stress in the gut. Using two-dimensional gel electrophoresis and mass spectrometry, we identified 22 up-regulated and 19 down-regulated protein spots in the jejunum of weanling piglets supplemented with ZnO (3,000 mg/kg Zn) compared with the control pigs (100 mg/kg Zn). These proteins are related to energy metabolism (increased level for succinyl-CoA transferase and decreased level for creatine kinase M-type); oxidative stress (decreased levels for 78 kDa glucose-regulated protein and glutathione-*S*-transferase-ω); and cell proliferation and apoptosis (increased levels for A-Raf-1 and calregulin). Consistent with the changes in protein expression, the ratio of reduced glutathione to oxidized glutathione was increased, whereas glutathione-*S*-transferase and vvglutathione peroxidase activities as well as the protein level of active caspase-3 were reduced in ZnO-supplemented piglets. Collectively, these results indicate that ZnO supplementation improves the redox state and prevents apoptosis in the jejunum of weaning piglets, thereby alleviating weaning-associated intestinal dysfunction and malabsorption of nutrients (including amino acids).

Keywords Apoptosis · Small intestine · Proteomics · Redox · ZnO

Abbreviations

ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
GRP78	78 kDa Glucose-regulated protein
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione-*S*-transferase
GSH-PX	Glutathione peroxidase
IEF	Isoelectric focusing
MEK	Mitogen-activated protein kinase kinase
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide electrophoresis

Introduction

The small intestine is the major organ involved in the digestion, absorption, and metabolism of dietary nutrients, including proteins and amino acids (Jobgen et al. 2006; Wu 1998). It also provides a defensive barrier against diet-derived pathogens, carcinogens and oxidants (Aw 2005). Post-weaning syndrome, manifested as anorexia, intestinal atrophy, diarrhea, and growth retardation in mammalian

X. Wang · J. Yin · G. Wu · J. Wang (✉)
State Key Laboratory of Animal Nutrition,
China Agricultural University, 100193 Beijing, China
e-mail: jkywjj@hotmail.com

D. Ou
College of Animal Science of Guizhou University,
550025 Guiyang, Guizhou, China

G. Wu
Department of Animal Science, Texas A&M University,
College Station, TX 77843, USA

neonates (including human infants), is a major problem in animal production and public health, especially in developing countries (Kong et al. 2007; Meier et al. 2003).

Zinc is an important dietary factor that regulates intestinal amino acid and protein metabolism in animals (Ou et al. 2007; Wang et al. 2008d). Previous studies with the weanling piglet have shown that dietary supplementation with ZnO (3,000 mg/kg Zn) enhanced intestinal mass (Li et al. 2006), inhibited stem-cell-factor expression in the jejunum (Ou et al. 2007), prevented diarrhea (Carlson et al. 1999), and improved growth (Hahn and Baker 1993; Hoque and Binder 2006; Yin et al. 2008). However, the underlying mechanisms are largely unknown. Because the piglet is widely used as an animal model to study human infant nutrition (Kim and Wu 2008; Suryawan et al. 2008), understanding how ZnO supplementation alleviates intestinal dysfunction in weanling piglets has important implications for both animal production and human medicine.

Our recent work shows that weaning results in increased expression of genes that promote oxidative stress and immune activation, decreases expression of genes related to nutrient utilization and cell proliferation, and increased the ratio of oxidized glutathione (GSSG) to reduced glutathione (GSH) in the piglet small intestine (Wang et al. 2008b). Therefore, we hypothesized that ZnO supplementation may affect expression of proteins related to glutathione metabolism and oxidative stress in the gut. The present study was designed to test this hypothesis using a two-dimensional gel electrophoresis/mass spectrometry proteomic approach (Wang et al. 2005, 2006), which provides a powerful discovery tool in nutrition research (Hu et al. 2008; Liu et al. 2008; Wang et al. 2008c).

Materials and methods

Experimental animals and diets

This experiment was approved by the China Agricultural University Animal Care and Use Committee. Thirty-two crossbred (Large White × Landrace × Pietran) barrows were weaned at 28 days of age (8.1 ± 0.6 kg BW) and allotted to two dietary treatments (normal vs. high levels of zinc) based on body weight and litter of origin. The basal diet contained the same composition as described by Yin et al. (2008) and was supplemented with 100 or 3,000 mg/kg zinc as ZnO. The diets were formulated to meet or exceed National Research Council requirements for all other nutrients (NRC 1998).

All weaned piglets were individually housed in 1.25 × 1.2 m stainless steel pens over a totally slotted floor. The pens were equipped with self-feeders and nipple drinkers in an environmentally controlled building (temperature maintained at 26–28°C with a 16-h light and 8-h dark cycle). Piglets had free access to feed and drinking water throughout the entire experiment. Weight gain and feed intake were recorded and used to calculate feed conversion during a 15-day period of the growth trial.

The incidence of diarrhea was recorded daily. The scoring was performed by two trained personnel with no prior knowledge of dietary treatment allocation. The fecal consistency was assessed as being either solid or liquid, with pigs excreting liquid feces being classified as having diarrhea. The incidence of diarrhea was expressed as a percentage, based on the number of pigs showing diarrhea each day and the total number of pigs on that treatment.

On day 15 of the trial, four piglets were randomly selected from each treatment group to obtain jejunal tissue samples. The number of piglets used was based on our recent studies of the proteome in newborn pigs (Wang et al. 2008a). Piglets were slaughtered by electrical stunning, followed by exsanguination. The abdomen was immediately exposed via a mid-line incision and the small intestine was removed. The small intestine in neonatal pig was defined as the portion of the digestive tract between the pylorus and the ileocecal valve, with the first 10-cm segment being duodenum (Wang et al. 2008b). The jejunum and ileum constituted approximately 40 and 60%, respectively, of the small intestine length below the duodenum (Wang et al. 2008b). A 10-cm portion was aseptically obtained from the middle portion of the jejunum, immediately placed in liquid nitrogen, and stored at −80°C until analyses.

Extraction of proteins

Proteins were extracted from the jejunum as we described (Wang et al. 2005, Yang et al. 2007). Briefly, 0.2 g of jejunal tissue (using the entire intestinal wall) was homogenized in a 450 μL lysis buffer (7 M solid urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, 50 mM dithiothreitol) containing 1% protease inhibitors (100×; Amersham Biosciences, Uppsala, Sweden). The protease inhibitor was specifically developed for sample preparation in two-dimensional gel electrophoresis studies to inhibit calpain II, cathepsin B, elastase, papain, plasmin, thermolysin and trypsin. Cells were ruptured at 0°C using a Model VCX 500 Ultrasonicater (Sonics & Materials, Newtown, CT) at 20% power output for 10 min with 2-s on and 8-s off cycles. After adding 1% (v/v) nuclease mix (Amersham Biosciences), the lysed cell suspension was kept at room temperature for 1 h to solubilize proteins (Tom and Tirra 1998), followed by re-sonication as described above to thoroughly break up cell membranes. The homogenate was subsequently

centrifuged for 10 min at 13,000×*g* at 15°C. The supernatant fluid was collected, and its protein concentration was determined using a PlusOne 2-D Quant Kit (Amersham Biosciences) and stored in aliquots of 1 mg protein at −80°C. The recovery of proteins was similar between the control and treatment groups.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis of the protein samples was run in quadruplicate. Isoelectric focusing (IEF) was performed using IPG strips (pH 3–10, nonlinear, 24-cm long; Amersham Biosciences). Samples (1 mg protein) were loaded onto IPG DryStrips using the in-gel sample rehydration technique, according to the manufacturer's instructions. The first-dimensional IEF was carried out at 20°C for 100,000-voltage hours in the IPGphor system (Amersham Biosciences), as described by Görg et al. (2000). After this step, each strip was equilibrated for 15 min in 4 mL equilibration buffer-1 (6 M urea, 1% dithiothreitol, 30% glycerol, and 50 mM Tris–Cl pH 8.8) and then in 4 mL equilibration buffer-2 (6 M urea, 2.5% iodoacetamide, 30% glycerol, and 50 mM Tris–Cl pH 8.8) for 15 min.

For the second dimensional IEF, vertical slab SAS-PAGE (12.5%) was run for 4 h at 50 mA per gel. The gel was then stained with colloidal Coomassie Brilliant Blue G-250 (Amresco Inc., Solon, OH). High-resolution gel images (400 dpi) were obtained using an ImageScanner Model PowerLook 2100XL (UMAX Technologies, Atlanta, GA) and image analysis was performed using an Image-Master 2D Platinum Version 6.01 (Amersham Biosciences). After normalizing the quantity of each spot by total valid spot intensity, we selected differentially expressed protein spots for determination of expression levels that significantly deviated ($P < 0.05$) over 1.3-fold in the relative volume (% vol), as recommended by Amersham Biosciences.

Identification of differentially expressed proteins

In-gel protein digestion, matrix assisted laser desorption ionization mass spectrometry and protein identification procedures were performed as described by Wang et al. (2008a). Peptide mass fingerprint searches were performed using the program MASCOT developed by Matrix Science Ltd (http:\www.matrixscience.com). Monoisotopic peptide masses were used to search the database, allowing a peptide mass accuracy of 300 ppm and one partial cleavage. Oxidation of methionine and carbamidomethyl modification of cysteine were considered as variable modifications. For protein identification by peptide mass fingerprint, peptide masses were searched against the mass spectrometry database for other mammals (Wang et al. 2008a).

Analysis of glutathione, glutathione-*S*-transferase (GST) and glutathione peroxidase (GSH-PX)

Jejunal tissue was analyzed for GSH and GSSG, as well as the activities GST and GSH-PX using kits supplied by the Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Results are expressed on the basis of tissue protein content.

Western blot for analysis of active caspase-3.

The active caspase-3 was determined by Western blot as described by Zhou et al. (2007). Briefly, the extracted protein sample (23 μg) was boiled for 5 min, electrophoresed (Bio-Rad, Richmond, CA) in 10% sodium dodecyl sulfate-polyacrylamide gel, and electroblotted (Bio-Rad) onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). After blocking with buffer containing 5% fat-free dry milk in TBST (0.05% Tween 20, 100 mM Tris–HCl and 150 mM NaCl, pH 7.5) for 1.5 h, the membrane was washed in TBST four times, and incubated for 2 h with a primary polyclonal antibody (1:2,000) for active caspase-3 (Cat # C8487, Sigma, St Louis, MO). The membrane was rinsed in TBST and incubated for 2 h with a secondary antibody (horseradish peroxidase-labeled anti-rabbit IgG diluted in 1:1,000) for anti-active caspase-3. The membrane was then washed three times with TBST. Glyceraldehyde-3-phophate dehydrogenase was used as the internal reference protein. The X-ray films were exposed using enhanced chemiluminescence to visualize the reactive bands, and then were scanned. The band density was quantified using a gel-imaging system (Tanon Science and Technology, Shanghai, China) with Image Analysis Software (National Institutes of Health, Bethesda, MD).

Statistical analysis

Normality of the data was tested using the Shapiro–Wilk test in SAS (version 8.1; SAS Institute, Cary, NC). Data were analyzed by one-way analysis of variance, with each animal as an experimental unit. All analyses were performed using SAS. Data are expressed as means and pooled SEM. A probability value ≤ 0.05 was taken to indicate statistical significance.

Results

Growth performance and diarrhea incidence of piglets

The growth performance of pigs fed diets containing a normal or high level of zinc is summarized in Table 1. Zn-supplemented piglets had a greater weight gain and feed

Table 1 Growth performance of weanling piglets fed diets containing a normal (100 mg/kg Zn) or high (3,000 mg/kg Zn) level of zinc

	Control	Treatment	SEM
Weight gain (g/day)	330	400**	16
Feed intake (g/day)	466	588*	27
Feed conversion	1.41	1.47	0.07

Data are mean ± SEM = 16

*$P < 0.05$ versus control; **$P < 0.01$ versus control

intake ($P < 0.05$), compared with the control piglets. Feed conversion was not affected by the level of dietary zinc. Pigs fed a high level of dietary zinc had few incidence of diarrhea, whereas pigs fed a normal level of zinc exhibited a high rate of diarrhea, which manifested itself primarily at day 5 after weaning (Fig. 1).

Differentially expressed proteins in jejunum

Results of the proteomic analysis revealed 22 up-regulated protein spots (>1.3-fold; $P < 0.05$) and 19 down-regulated protein spots (>1.3-fold; $P < 0.05$) in the jejunum of ZnO-supplemented piglets, compared with the control piglets (Tables 2, 3). Biochemical information about these protein spots is summarized in Tables 2 and 3, whereas their appearance on the images is labeled in Figs. 2 and 3. Among these protein spots, 11 and 14 of them were found only in the treatment or control groups, respectively, while the remainder were detected in both treatments. Dietary supplementation with ZnO increased ($P < 0.05$) the concentrations of succinyl-CoA transferase, A-Raf-1, and calregulin, while reducing ($P < 0.05$) the levels of creatine kinase M-type, 78 kDa glucose-regulated protein (GRP78), and GST-ω, in comparison with the control group (Fig. 4).

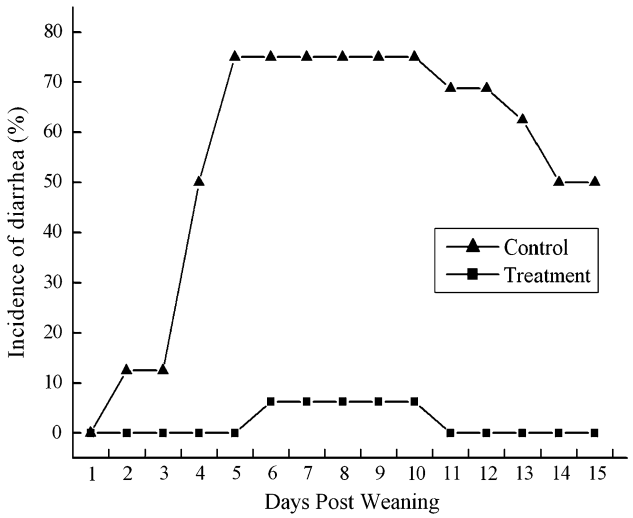

Fig. 1 Diarrhea incidence in pigs fed normal (100 mg/kg Zn) or high (3,000 mg/kg Zn) levels of zinc oxide

Changes of redox state, apoptosis and related enzymes

The results of the proteomics analysis indicate an improvement in the redox state of the ZnO-supplemented piglet jejunum. To provide direct evidence to substantiate this observation, we determined the activities of oxidative stress-related proteins (GST and GSH-PX) and concentrations of GSH and GSSG in the jejunum. Dietary supplementation with ZnO did not affect GSH concentration but markedly reduced ($P < 0.01$) GSSG concentration, resulting in an 80% increase of the GSH:GSSG ratio in the jejunum (Table 4). Jejunal activities of GST and GSH-PX were 81 and 47% lower ($P < 0.05$), respectively, in ZnO-supplemented piglets than in the control group (Table 4). Similarly, dietary supplementation with ZnO reduced ($P < 0.05$) the concentration of jejunal active caspase-3 (an *apoptosis-related marker protein*) by 61%, compared with the control group (Fig. 5).

Discussion

Supplementing a high level of ZnO (e.g., 3,000 mg Zn/kg in diet) to the diet of weanling piglets reduces the incidence of diarrhea and enhances growth performance, therefore providing a therapeutic strategy against post-weaning syndrome (Carlson et al. 1999; Hahn and Baker 1993; Ou et al. 2007). In addition, results of the current work indicate that Zn supplementation increased body-weight gain and reduced the incidence of diarrhea in weanling piglets, possibly by regulating cellular signaling pathways (Liao et al. 2008; Rhoads and Wu 2008). Moreover, using the high throughput technology of proteomics (Capone et al. 2008; Zhao et al. 2008; Yan and He 2008), we found that ZnO favorably increased the expression of anti-oxidative proteins, reduced the expression of apoptotic proteins (Tables 2, 3), and improved the redox state as indicated by an increase in the GSH:GSSG ratio in the jejunum of the weanling piglet (Table 4). These findings provide new knowledge about an important role for ZnO in regulating intestinal glutathione metabolism and redox signaling.

A novel and important finding of this study is that supplementing ZnO to the piglet diet increased the expression of succinyl-CoA transferase (Table 2), while decreasing the expression of creatine kinase in the jejunum (Table 3). Succinyl-CoA transferase transfers CoA from succinyl-CoA to acetoacetate via a thioester intermediate to yield succinate and acetoacetyl-CoA, whereas creatine kinase converts creatine and ATP into phosphocreatine. Therefore, these two proteins are related to energy

Table 2 Biochemical information about proteins whose expression was increased in the jejunum of ZnO-supplemented piglets

Spot number	Accession code	Protein name	Ratio (treatment/control)	Score[a]	Sequence coverage (%)	No. of matched peptides (observed/predicted)	Protein functional category
1	gil47522610	Succinyl-CoA-transferase	OT	81	34	11/38	Cellular ketone body metabolic process
2	gil55742847	A-Raf-1	OT	71	18	9/23	Intracellular signaling cascade
3	gil75048592	Arginine ADP-ribosyltransferase 5	OT	63	40	7/44	Protein amino acid ADP-ribosylation
4	gil45269029	Cytoskeletal beta actin	OT	136	56	21/57	Cytoskeleton
5	gil1890664	Glucosidase II	OT	117	28	19/48	Carbohydrate metabolic process
6	gil49355314	Immunoglobulin IgG-1	OT	76	24	6/33	Antigen binding
7	gil410689	Leucyl aminopeptidase	OT	65	23	10/45	Protein metabolic process
8	gil27807355	$NADH_2$ dehydrogenase 75 K chain precursor	OT	67	22	12/34	NADH dehydrogenase activity
9	gil160011348	Mannose-1-phosphate guanyltransferase beta	OT	90	37	12/30	Biosynthetic process
10	gil71152981	Serum albumin precursor	OT	89	38	17/56	Transport
11	gil66954256	Unnamed protein product	OT	74	27	10/28	Unknown
12	gil75060993	Alpha3-fucosyltransferase	2.14	62	14	6/27	Protein amino acid glycosylation
13	gil75046546	Beta actin	1.42	87	58	15/60	Cytoskeleton
14	gil75062970	Cytoskeletal beta actin	1.82	101	42	14/53	Cytoskeleton
15	gil75038971	Very low density lipoprotein receptor	1.72	63	25	16/53	Receptor activity
16	gil231467	Fetuin precursor	1.50	97	44	11/36	Positive regulation of phagocytosis
17	gil117505	Calregulin	2.10	104	63	10/84	Metal ion binding
18	gil71152981	Serum albumin precursor	1.40	241	42	23/31	Transport
19	gil5597005	Beta actin	1.40	94	46	11/47	Cytoskeleton
20	gil75062970	Cytoskeletal beta actin	1.40	104	45	18/56	Cytoskeleton
21	gil122138156	Alpha thalassemia mental retardation Y homolog	1.33	81	13	17/32	Zinc ion binding
22	gil122135995	Lin-7 homolog B	1.31	76	32	7/69	Protein transport

OT only detected in the treatment group

[a] Protein score is $-10 \times \log(P)$ where P is the probability that the observed match is a random event. Protein scores of 59 or greater indicate a significant match ($P < 0.05$) with the named protein

metabolism, which undergoes marked changes in response to stress (Sims 1996; Wu et al. 2007, 2008b). Because pigs have a limited capacity for ketogenesis (Duee et al. 1994), it is unlikely that succinyl-CoA transferase would function primarily for the utilization of ketone bodies in pigs. However, the formation of succinate would allow for the oxidation of acetyl-CoA derived from amino acids, glucose, and fatty acids in the small intestine (Jobgen et al. 2006; Wu 1998). Additionally, creatine kinase plays an important role in ATP utilization by contracting muscles (e.g., smooth muscle in the gut), whereas a low level of this protein in the small intestine may reduce the conversion of ATP to ADP (Gualano et al. 2008; Wu et al. 2008a), therefore making ATP available for maintenance of high rates of protein turnover and cell proliferation, as well as barrier integrity (Wang et al. 2008d).

Another salient observation from the present study was that the expression of proteins related to oxidative defense was beneficially altered in the jejunum of ZnO-supplemented piglets. Weaning stress, which is provoked by abrupt social, dietary and environmental changes, as well as anorexia, digestive dysfunction, and impaired immune response, is associated with oxidative injury in the small intestine (Wang et al. 2008b). As the primary defense barrier against diet-derived mutagens, carcinogens and oxidants, optimal development of anti-oxidative capacity

Table 3 Biochemical information about proteins whose expression was reduced in the jejunum of ZnO-supplemented piglets

Spot number	Accession code	Protein name	Ratio (treatment/control)	Score[a]	Sequence coverage (%)	No. of matched peptides (observed/predicted)	Protein functional category
23	gil14916993	78 kDa glucose-regulated protein	−1.89	80	36	9/48	Endoplasmic reticulum
24	gil122140860	Lymphocyte cytosolic protein 1	−1.54	154	31	20/34	Phagocytic cup
25	gil122142568	55 kDa erythrocyte membrane protein	−1.44	68	34	15/75	Membrane
26	gil30315379	Proteasome activator 28 alpha subunit	−1.92	59	31	7/49	Proteasome activator activity
27	gil122134663	Proteasome beta 10 subunit	−4.10	72	35	8/30	Ubiquitin-dependent protein catabolic process
28	gil132574	Ribonuclease inhibitor	OC[1]	71	31	8/21	Protein binding
29	gil1703236	Alcohol dehydrogenase [NADP+]	OC	76	32	9/44	Oxidoreductase
30	gil62286641	Creatine kinase M-type	OC	78	34	10/29	Transferase activity, transferring phosphorus-containing groups
31	gil75057767	Target of myb1	OC	60	16	7/28	Intracellular protein transport
32	gil6166115	Desmin	OC	59	29	11/51	Intermediate filament
33	gil3660252	F1-ATPase delta and 1 epsilon subunits	OC	79	26	10/28	ATP biosynthetic process
34	gil12249197	Glutathione-*S*-transferase ω	OC	152	66	16/46	Glutathione transferase activity
35	gil86826556	Histidyl-tRNA synthetase-like	OC	65	28	10/38	Translation
36	gil88930442	Histone-binding protein RBBP4	OC	124	41	13/30	Regulation of transcription, DNA-dependent
37	gil41323529	Immunoglobulin kappa variable region	OC	70	54	8/62	Immune response
38	gil27807161	Isocitrate dehydrogenase (NAD) chain 3/4 precursor	OC	143	47	24/51	Tricarboxylic acid cycle
39	gil90110026	Keratin, type II cytoskeletal 8	OC	149	36	21/51	Apoptosis
40	gil127173	Myosin regulatory light chain	OC	98	62	12/30	Myosin complex
41	gil11968062	Phosphoprotein phosphatase 1 catalytic chain	OC	174	53	16/23	Phosphoprotein phosphatase activity

OC only detected in the control group

[a] Protein score is $-10 \times \log(P)$ where P is the probability that the observed match is a random event. Protein scores of 59 or greater indicate a significant match ($P < 0.05$) with the named protein

during weaning is crucial for health and growth (Wu et al. 2008b). We found that protein levels for GRP78 and GST-ω, which are associated with oxidative stress (Lee 2005), were reduced in the jejunum of ZnO-supplemented piglets (Fig. 4). This result is consistent with an elevated GSH:GSSG ratio and reduced enzymatic activities of GST and GSH-PX in the jejunum of ZnO-supplemented piglets (Table 4).

GRP78, an immunoglobulin heavy-chain binding protein, is a member of the 70 kDa heat shock protein family (Lee 2001). Recent work has shown that GRP78 is a master regulator of endoplasmic reticulum (ER) function due to its role in (1) protein folding and assembly; (2) targeting misfold protein for degradation; (3) Ca^{2+} binding in ER; and (4) controlling the activation of transmembrane ER stress sensors (i.e. inositol-requiring enzyme 1, IRE1; PKR-like ER kinase and transcription factor 6) (Lee 2005). Hence, up-regulation of GRP78 has been wildly used as a marker for ER stress and the presence of unfolded proteins (Sun et al. 2006). Thus, GRP78 expression is greatly enhanced under a variety of stressful conditions, including environmental and nutritional stresses (e.g., glucose deprivation, inhibition of glycosylation, treatment with Ca^{2+} ionophores, and deficiency of an essential amino acid), oxidative stress, and hypoxia (Baek et al. 2004; Kaufman 1999). Additionally, GST-ω is present in all living organisms and its primary function is the detoxification of electrophilic compounds via transferring the glutathione moiety to a variety of acceptor molecules, including toxins, organic hydroperoxides, and lipid

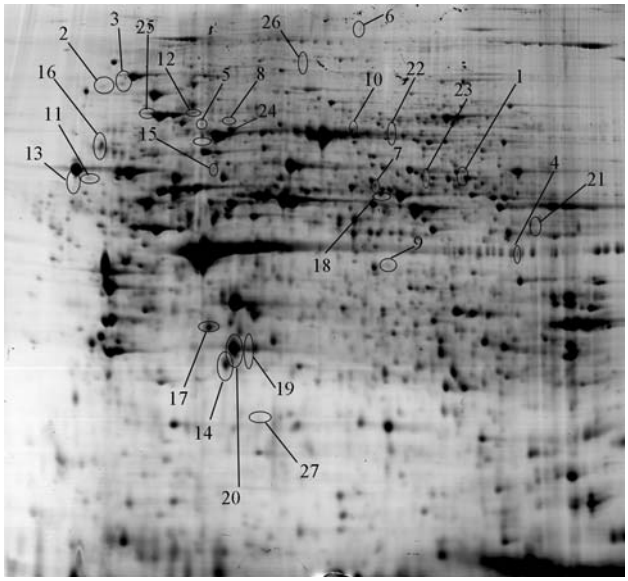

Fig. 2 Two-dimensional gel electrophoresis showing the distribution of differently expressed proteins from the jejunum of piglets fed a diet supplemented with high levels of zinc oxide (3,000 mg/kg)

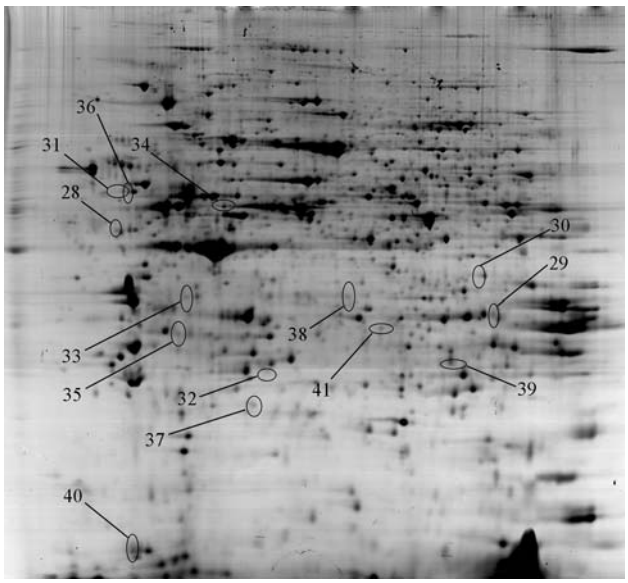

Fig. 3 Two-dimensional gel electrophoresis showing the distribution of differently expressed proteins from the jejunum of piglets fed a diet containing a normal level of zinc oxide (100 mg/kg)

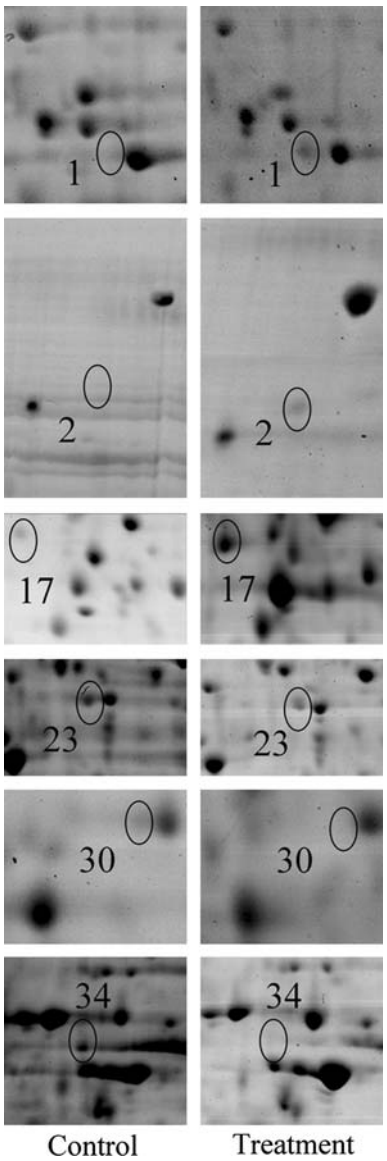

Fig. 4 Abundance of partial differentially expressed proteins from the jejunum of piglets fed a diet containing a normal (100 mg/kg) or high level (3,000 mg/kg) of zinc oxide. *1* Succinyl-CoA transferase, *2* A-Raf-1, *17* calregulin, *23* 78 kDa glucose-regulated protein; *30* creatine kinase M-type, *34* glutathione-*S*-transferase ω

peroxides to form conjugates (Fang et al. 2002; Rouimi et al. 2001). Therefore, GST is crucial in the GSH redox cycle, which is a major pathway for peroxide elimination in the small intestine (Aw 2005). When levels of oxidative stress are high, proteins are oxidized (Galli 2007; Ptolemy et al. 2007) and GST expression is usually induced (Zhu et al. 2006). A decrease in both GRP78 and GST-ω proteins indicates a reduced level of intestinal oxidative stress in the jejunum of ZnO-supplemented piglets.

Glutathione plays an important cytoprotective role in the gastrointestinal tract against damage from insults such as radiation, endotoxins, and reactive oxygen species (Rider et al. 2007; Wu et al. 2004). The GSH:GSSG ratio is often used as an index of the redox state in tissues (Higashida et al. 2009; Manna et al. 2008). Elevation of both GST and GSH-PX activities may be an adaptive mechanism secondary to the increase of oxidative stress (Mannick 2007; Zhu et al. 2006). Consistent with this notion, we found that dietary supplementation with ZnO increased the GSH:GSSG ratio in the small intestine (Table 4).

Table 4 Concentrations of GSH and GSSG, as well as GST and GSH-PX activities in the jejunum of weanling piglets

	GSH μmol/g protein	GSSG	GSH/GSSG %	GST U/mg protein	GSH-PX
Control	702.8 ± 36.0	9.12 ± 0.66	77.7 ± 3.2	97.2 ± 9.3	156.9 ± 33.5
Treatment	609.7 ± 40.8	4.59 ± 0.76**	142.8 ± 24.5*	51.4 ± 8.7*	29.8 ± 3.4**

Values are mean ± SEM, $n = 4$

*$P < 0.05$ versus control; **$P < 0.01$ versus control

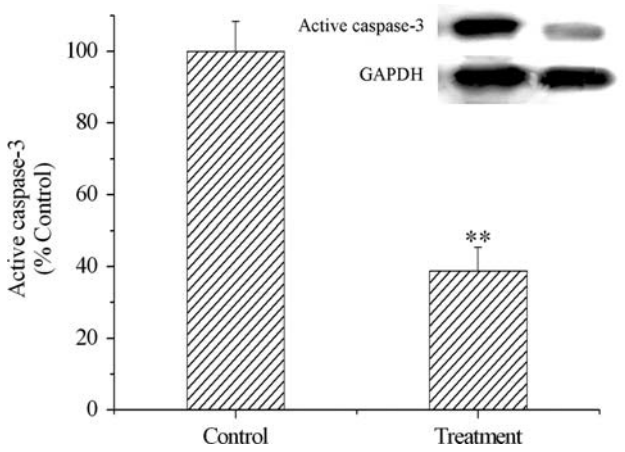

Fig. 5 Western blot analysis of active caspase-3 in the jejunum of weanling piglets fed a diet supplemented with a normal (100 mg/kg) or high level (3,000 mg/kg) of zinc oxide. GAPDH was used as the internal reference protein. Values are mean ± SEM, $n = 4$. **$P < 0.05$ versus control

Another novel and exciting result of the current study is that ZnO supplementation increased expression of A-Raf-1 and calregulin (Table 2), while decreasing expression of active caspase-3 in the piglet jejunum (Fig. 5). A-Raf-1 (A-Raf proto-oncogene serine/threonine-protein kinase) can activate tyrosine kinase via serine-threonine phosphorylation and also links growth factor receptors with transcription factors (Gerwins et al. 1997). This signal-integrating enzyme belongs to the mammalian Raf family (A-raf, B-raf, and C-raf) of proteins, which are the principal effectors of Ras in the mitogen-activated protein kinase pathway (Beeram et al. 2005). Mitogen-activated protein kinase activates a kinase cascade of Raf/MEK/ERK to propagate signaling of cell proliferation from the plasma membrane to the nucleus (Phang et al. 2008; Zhang et al. 2008). In addition, A-Raf-1 has an antiapoptotic effect through its physical interaction (phosphorylation and inactivation) with the apoptosis-promoting B cell lymphoma 2-associated "death" promoter (Beeram et al. 2005). Additionally, calregulin, also named calreticulin, is a unique ER luminal resident protein with multiple functions, including chaperoning as well as regulation of both Zn^{2+} and Ca^{2+} levels in synaptic vesicles (Michalak et al. 1999). Indeed, up-regulation of calregulin is associated with increases in protein synthesis and cell proliferation (Opas et al. 1991). Such a role for calregulin may contribute to enhancement of intestinal cell growth and mucosal mass in ZnO-supplemented piglets (Ou et al. 2007; Yin et al. 2008). Moreover, active caspase-3, an apoptosis marker, is positively correlated with cell apoptosis (Chandra et al. 2000). Our finding provides a molecular basis to explain the previous report that zinc plays an important role in inhibiting apoptosis (Formigari et al. 2007).

Weanling piglets often exhibit intestinal atrophy and dysfunction (Wu et al. 1996). Thus, supplementing a pharmacological level of ZnO to the diet is highly relevant to the management of neonatal pigs under production conditions. We are not aware of its use for infants or children. However, oral administration of $ZnSO_4$ (3–20 mg Zn/kg body weight) has been reported to reduce the duration as well as the severity of acute and persistent diarrhea in children (Bhutta et al. 2000). Additionally, a recent study has shown that oral administration of $ZnSO_4$ can prevent diarrhea and enhance growth in 1- to 6-month-old human infants (Bhandari et al. 2008). Results of the current study with piglets not only aid in elucidating the mechanisms responsible for the beneficial effect of Zn on neonates but also guide future work to define the cellular action of $ZnSO_4$. Understanding how dietary supplementation with ZnO alleviates diarrhea in piglets has important implications for human nutrition and medicine.

In conclusion, the results of this proteomic study indicate that dietary supplementation with ZnO beneficially alters the expression of intestinal proteins that are related to the regulation of oxidative stress, redox state, cell proliferation, and apoptosis in weanling piglets. These novel findings provide a molecular basis for the anti-oxidative function of zinc and a new framework to study the mechanism responsible for the therapeutic effect of Zn on intestinal function in neonates.

Acknowledgments The research was financially supported by the National Natural Science Foundation of China (no. 30600434, 30571343, and 30671517) and Texas AgriLife Research (H#8200).

References

Aw TY (2005) Intestinal glutathione: determinant of mucosal peroxide transport, metabolism, and oxidative susceptibility. Toxicol Appl Pharmacol 204:320–328

Baek HY, Lim JW, Kim H et al (2004) Oxidative-stress-related proteome changes in *Helicobacter pylori*-infected human gastric mucosa. Biochem J 379:291–299

Beeram M, Patnaik A, Rowinsky EK (2005) Raf: a strategic target for therapeutic development against cancer. J Clin Oncol 23:6771–6790

Bhandari N, Mazumder S, Taneja S et al (2008) Effectiveness of zinc supplementation plus oral rehydration salts compared with oral rehydration salts alone as a treatment for acute diarrhea in a primary care setting: a cluster randomized trial. Pediatrics 121:e1279–e1285

Bhutta ZA, Bird SM, Black RE et al (2000) Therapeutic effects of oral zinc in acute and persistent diarrhea in children in developing countries: pooled analysis of randomized controlled trials. Am J Clin Nutr 72:1516–1522

Capone G, De Marinis A, Simone S et al (2008) Mapping the human proteome for non-redundant peptide islands. Amino Acids 35:209–216

Carlson MS, Hill GM, Link JE (1999) Early- and traditionally weaned nursery pigs benefit from phase-feeding pharmacological concentrations of zinc oxide: effect on metallothionein and mineral concentrations. J Anim Sci 77:1199–1207

Chandra J, Samali A, Orrenius S (2000) Triggering and modulation of apoptosis by oxidative stress. Free Radic Biol Med 29:323–333

Duee PH, Pegorier JP, Quant PA et al (1994) Hepatic ketogenesis in newborn pigs is limited by low mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase activity. Biochem J 298:207–212

Fang YZ, Yang S, Wu G (2002) Free radicals, antioxidants, and nutrition. Nutrition 18:872–879

Formigari A, Santon A, Irato P (2007) Efficacy of zinc treatment against iron-induced toxicity in rat hepatoma cell line H4-II-E-C3. Liver Int 27:120–127

Galli F (2007) Amino acid and protein modification by oxygen and nitrogen species. Amino Acids 32:497–499

Gerwins P, Blank JL, Johnson GL (1997) Cloning of a novel mitogen-activated protein kinase kinase kinase, MEKK4, that selectively regulates the c-Jun amino terminal kinase pathway. J Biol Chem 272:8288–8295

Görg A, Obermaier C, Boguth G et al (2000) The current state of two-dimensional electrophoresis with immobilized pH gradients. Electrophoresis 21:1037–1053

Gualano B, Novaes RB, Artioli GG et al (2008) Effects of creatine supplementation on glucose tolerance and insulin sensitivity in sedentary healthy males undergoing aerobic training. Amino Acids 34:245–250

Hahn JD, Baker DH (1993) Growth and plasma zinc responses of young pigs fed pharmacologic levels of zinc. J Anim Sci 71:3020–3024

Higashida M, Xu S, Kojima-Yuasa A et al (2009) 1′-Acetoxychavicol acetate-induced cytotoxicity is accompanied by a rapid and drastic modulation of glutamine metabolism. Amino Acids 36:107–113

Hoque KM, Binder HJ (2006) Zinc in the treatment of acute diarrhea: current status and assessment. Gastroenterology 130:2201–2205

Hu CA, Khalil S, Zhaorigetu S et al (2008) Human Δ^1-pyrroline-5-carboxylate synthase: function and regulation. Amino Acids 35:665–672

Jobgen WS, Fried SK, Fu WJ et al (2006) Regulatory role for the arginine-nitric oxide pathway in metabolism of energy substrates. J Nutr Biochem 17:571–588

Kaufman RJ (1999) Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev 13:1211–1233

Kim SW, Wu G (2008) Regulatory role for amino acids in mammary gland growth and milk synthesis. Amino Acids. doi: 10.1007/s00726-008-0151-5

Kong XF, Wu GY, Liao YP et al (2007) Effects of Chinese herbal ultra-fine powder as a dietary additive on growth performance, serum metabolites and intestinal health in early-weaned piglets. Livest Sci 108:272–275

Lee AS (2001) The glucose-regulated proteins: stress induction and clinical applications. Trends Biochem Sci 26:504–510

Lee AS (2005) The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. Methods 35:373–381

Li X, Yin J, Li D et al (2006) Dietary supplementation with zinc oxide increases Igf-I and Igf-I receptor gene expression in the small intestine of weanling piglets. J Nutr 136:1786–1791

Liao XH, Majithia A, Huang XL, Kimmel AR (2008) Growth control via TOR kinase signaling, an intracellular sensor of amino acids and energy availability, with crosstalk potential to proline metabolism. Amino Acids 35:761–770

Liu ZP, Wu LY, Wang Y et al (2008) Bridging protein local structures and protein functions. Amino Acids 35:627–650

Manna P, Sinha M, Sil PC (2008) Taurine plays a beneficial role against cadmium-induced oxidative renal dysfunction. Amino Acids. doi: 10.1007/s00726-008-0094-x

Mannick JB (2007) Regulation of apoptosis by protein *S*-nitrosylation. Amino Acids 32:523–526

Meier R, Burri E, Steuerwald M (2003) The role of nutrition in diarrhoea syndromes. Curr Opin Clin Nutr Metab Care 6:563–567

Michalak M, Corbett EF, Mesaeli N et al (1999) Calreticulin: one protein, one gene, many functions. Biochem J 344:281–292

Opas M, Dziak E, Fliegel L, Michalak M (1991) Regulation of expression and intracellular distribution of calreticulin, a major calcium binding protein of nonmuscle cells. J Cell Physiol 149:160–171

Ou D, Li D, Cao Y et al (2007) Dietary supplementation with zinc oxide decreases expression of the stem cell factor in the small intestine of weanling pigs. J Nutr Biochem 18:820–826

Phang JM, Donald SP, Pandhare J, Liu YM (2008) The metabolism of proline, a stress substrate, modulates carcinogenic pathways. Amino Acids 35:681–690

Ptolemy AS, Lee R, Britz-McKibbin P (2007) Strategies for comprehensive analysis of amino acid biomarkers of oxidative stress. Amino Acids 33:3–18

RCN (1998) Nutrients requirements of swine. National Academy Press, Washington DC

Rhoads JM, Wu G (2008) Glutamine, arginine, and leucine signaling in the intestine. Amino Acids. doi: 10.1007/s00726-008-0225-4

Rider JE, Hacker A, Mackintosh CA et al (2007) Spermine and spermidine mediate protection against oxidative damage caused by hydrogen peroxide. Amino Acids 33:231–240

Rouimi P, Anglade P, Benzekri A et al (2001) Purification and characterization of a glutathione *S*-transferase Omega in pig: evidence for two distinct organ-specific transcripts. Biochem J 358:257–262

Sims NR (1996) Energy metabolism, oxidative stress and neuronal degeneration in Alzheimer's disease. Neurodegeneration 5:435–440

Sun FC, Wei S, Li CW et al (2006) Localization of GRP78 to mitochondria under the unfolded protein response. Biochem J 396:31–39

Suryawan A, O'Connor PMJ, Bush JA et al (2008) Differential regulation of protein synthesis by amino acids and insulin in

peripheral and visceral tissues of neonatal pigs. Amino Acids. doi: 10.1007/s00726-008-0149-z
Tom B, Tirra S (1998) 2-D electrophoresis: using immobilized pH gradients. Principles & Methods. Amersham Pharmacia Biotech, Uppsala
Wang J, Ying T, Wang H et al (2005) 2-D reference map of *Bacillus anthracis* vaccine strain A16R proteins. Proteomics 5:4488–4495
Wang J, Li D, Dangott LJ, Wu G (2006) Proteomics and its role in nutrition research. J Nutr 136:1759–1762
Wang J, Chen L, Li D et al (2008a) Intrauterine growth restriction affects the proteomes of the small intestine, liver, and skeletal muscle in newborn pigs. J Nutr 138:60–66
Wang J, Chen L, Li P et al (2008b) Gene expression is altered in piglet small intestine by weaning and dietary glutamine supplementation. J Nutr 138:1025–1032
Wang J, Wu G, Zhou H, Wang F (2008c) Emerging technologies for amino acid nutrition research in the post-genome era. Amino Acids. doi: 10.1007/s00726-008-0193-8
Wang W, Qiao S, Li DF (2008d) Amino acids and gut function. Amino Acids. doi: 10.1007/s00726-008-0152-4
Wu G (1998) Intestinal mucosal amino acid catabolism. J Nutr 128:1249–1252
Wu G, Meier SA, Knabe DA (1996) Dietary glutamine supplementation prevents jejunal atrophy in weaned pigs. J Nutr 126:2578–2584
Wu G, Fang YZ, Yang S et al (2004) Glutathione metabolism and its implications for health. J Nutr 134:489–492
Wu G, Bazer FW, Davis TA et al (2007) Important roles for the arginine family of amino acids in swine nutrition and production. Livest Sci 112:8–22
Wu G, Bazer FW, Datta S et al (2008a) Proline metabolism in the conceptus: implications for fetal growth and development. Amino Acids 35:691–702
Wu G, Bazer FW, Davis TA et al (2008b) Arginine metabolism and nutrition in growth, health and disease. Amino Acids. doi: 10.1007/s00726-008-0210-y
Yan GR, He QY (2008) Functional proteomics to identify critical proteins in signal transduction pathways. Amino Acids 35:267–274
Yang F, Wang J, Li X et al (2007) 2-DE and MS analysis of interactions between *Lactobacillus fermentum* I5007 and intestinal epithelial cells. Electrophoresis 28:4330–4339
Yin J, Li X, Li D et al (2008) Dietary supplementation with zinc oxide stimulates ghrelin secretion from the stomach of young pigs. J Nutr Biochem doi:10.1016/j.jnutbio.2008.07.007
Zhang SW, Zhang YL, Yang HF et al (2008) Using the concept of Chou's pseudo amino acid composition to predict protein subcellular localization: an approach by incorporating evolutionary information and von Neumann entropies. Amino Acids 34:565–572
Zhao XM, Chen LN, Aihara K (2008) Protein function prediction with high-throughput data. Amino Acids 35:517–530
Zhou X, Li D, Yin J et al (2007) CLA differently regulates adipogenesis in stromal vascular cells from porcine subcutaneous adipose and skeletal muscle. J Lipid Res 48:1701–1709
Zhu Y, Carvey PM, Ling Z (2006) Age-related changes in glutathione and glutathione-related enzymes in rat brain. Brain Res 1090:35–44