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Soybean-derived β -conglycinin affects proteome expression in pig intestinal cells in vivo and in vitro¹

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ABSTRACT: It is well known that β -conglycinin, a soybean allergen, induces allergies and causes intestinal damage in fetuses and neonates. However, the underlying mechanisms responsible for the adverse effects of β -conglycinin remain elusive. In particular, it is unknown whether or not this dietary substance causes direct damage affecting the proliferation and integrity of intestinal cells. This study evaluated the effect of different concentrations of β -conglycinin (0 to 1,500 $\mu\text{g/mL}$) and the duration of culture (48 or 72 h) on the proliferation and proteome of porcine intestinal epithelial cells. Eight individually housed piglets (10 d old; initial BW, 3.79 ± 0.07 kg) were randomly divided into 2 groups ($n = 4$) and challenged with or without β -conglycinin via oral administration d 10 through 28. After the last

administration of β -conglycinin or PBS, piglets were killed and jejuna mucosal samples were collected for proteomic analysis. Supplementing β -conglycinin to either culture medium or weanling pigs increased ($P < 0.05$) the expression of proteins related to apoptosis, stress, and inflammation, but decreased ($P < 0.05$) the expression of proteins related to cytoskeleton and nucleus replication in intestinal cells. Further analysis confirmed an increase in caspase-3 expression in the cells exposed to β -conglycinin in vivo and in vitro. Collectively, these novel results indicate that β -conglycinin directly induces intestinal damage by depressing intestinal-cell growth, damaging the cytoskeleton, and causing apoptosis in the piglet intestine.

Key words: allergy, apoptosis, β -conglycinin, intestine, pig, proteome

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INTRODUCTION

Soybeans are a high-quality source of protein and oil for animals (Friedman and Brandon, 2001). However, the presence of allergenic components in soybeans greatly restricts their use in diets for livestock species, particularly neonatal and weanling pigs (L'Hocine and Boye, 2007). Soybean allergies usually cause inflammatory disorders in the small intestine, characterized by villous atrophy and hyperplasia in the crypt, as well as

increased intestinal membrane permeability (Li et al., 1990). Furthermore, diarrhea, vomiting, and failure to thrive have been observed in some allergic cases, especially in infants and young mammals (Sicherer, 2005). Soybean allergies can also cause inflamed intestinal lining and damage the microvilli, therefore reducing production and secretion of digestive enzymes. The villous atrophy results in malabsorption, leading to reduced growth and increased incidence of other intestinal diseases, such as enteritis (Stokes et al., 1987). Although the adverse effects of soybean allergy are extensive and severe, the underlying mechanisms through which it manifests these changes are largely unknown.

Intestinal epithelial cells, located at the first layer of the intestine (Wang et al., 2009b), play an important role in separating the harsh luminal environment from the interior milieu (Oswald, 2006). However, the pathological uptake of bacterial products, toxins, and other allergens not only activates immune cells, but also directly affects intestinal epithelial cell function (Ruemmele et al., 2002). We hypothesized that β -conglycinin, a major allergen protein found in soybeans (Guo et al., 2007; Hao et al., 2009; Holzhauser et al., 2009) can ad-

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versely affect intestinal epithelial cells and promote allergy. In the present research, this hypothesis was tested in vivo and in vitro using the piglet model (Suryawan et al., 2009; Tan et al., 2009) and proteomic technology (Wang et al., 2006, 2009a, 2009c, 2010).

MATERIALS AND METHODS

This experiment was approved by the China Agricultural University Institutional Animal Care and Use Committee.

Porcine Intestinal Epithelial Cell Culture

Porcine intestinal epithelial cells (IPEC) were provided by Texas A&M University (Haynes et al., 2009; Tan et al., 2010). The cells were maintained at 37°C and 5% CO₂ in media (Dulbecco's Modified Eagle Media: Nutrient Mixture F-12, Gibco, Grand Island, NY) supplemented with 5% fetal calf serum (Gibco), 100 IU/mL of penicillin, and 100 IU/mL of streptomycin. The culture medium was changed every other day.

Effect of β -Conglycinin on IPEC Proliferation

The IPEC were allowed to attach overnight (Corning-Costar 96 multi-well plates, Sigma, St. Louis, MO) in complete medium containing 5% fetal calf serum. After a 22-h stabilization period in 1% complete medium, the cells were incubated with 0, 80, 160, 750, or 1,500 μ g/mL of β -conglycinin for 48 or 72 h. The purified β -conglycinin was provided by the Food Institute of China Agricultural University (Beijing, China) and was further purified through a 2.5 $\times$ 100 cm column (Sephacrose CL-6B, Sigma). The purity of β -conglycinin was judged to be over 95% according to SDS-PAGE analysis (Labwork 4.6, Media Cybernetics Inc., Bethesda, MD). At the end of the treatment period, reagents (Cell-Counting Kit-8 reagents, Dojindo Laboratories, Kumamoto, Japan) were added to the medium, and the optical density of the medium was determined at 450 nm and converted to cell numbers according to a calibration curve.

Treatment of IPEC for Proteomic Analysis

The IPEC were maintained and stabilized in 1% complete medium for 22 h and were then cultured in 1% complete medium with or without 160 μ g/mL of β -conglycinin for an additional 72 h. The cells were then harvested for proteomic analysis.

Piglet Feeding and Collection of Mucosal Samples

Eight 10-d-old piglets (Large White $\times$ Landrace), with an initial BW of 3.79 ± 0.07 kg, were used for

the present study. These pigs were the same as those used in the work of Hao et al. (2009). All piglets were individually housed in 1.3 $\times$ 1.2 m pens with slatted stainless-steel floors. The mechanically ventilated nursery room was subjected to a photoperiod of 16-h light and 8-h dark, and its temperatures were maintained at 25 to 27°C. A soybean- and peanut-free diet containing skim milk powder and casein was formulated to meet or exceed nutrient requirements for young pigs and contained no antibiotics (Table 1). The content of nutrients in the diet was analyzed according to AOAC procedures. Gross energy was determined by an automatic adiabatic oxygen bomb calorimeter (Parr 1281 Automatic Energy Analyzer, Parr Instrument Co., Moline, IL). Phosphorus content was analyzed using the UV-visible spectrophotometer (U-1000, Hitachi, Tokyo, Japan). The piglets were randomly divided into 2 groups (control and β -conglycinin treatment). The β -conglycinin was dissolved in PBS. Piglets in the treatment group were sensitized by oral administration of β -conglycinin (2 g/piglet) daily between d 0 and 6, followed by boosting with 8 g of β -conglycinin/piglet on d 21 and 12 g of β -conglycinin/piglet on d 28. Piglets in the control group were gavaged with the same volume of PBS. Three hours after the last administration of β -conglycinin or PBS, piglets were killed with an intracardial injection of sodium pentobarbital (50 mg/kg of BW) followed by exsanguination. The abdomen was immediately exposed via a midline incision. The middle jejunum was removed and washed with saline. Then the whole mucosa of the middle jejunum was removed using glass slides and stored at -80°C until analysis (Wang et al., 2009c, 2010).

Protein Extraction

The IPEC obtained from the in vitro study and jejunal mucosa from the in vivo study were used for protein extraction as described previously (Yang et al., 2007; Wang et al., 2008). Briefly, IPEC pellets were washed 3 times with ice-cold PBS and then centrifuged for 10 min at $350 \times g$ at 4°C. Approximately 0.2 g of cells or mucosa were homogenized in a lysis buffer containing 7 M urea, 2 M thiourea, 4% 3-[3-(cholamidopropyl)-dimethylammonio]-1-propanesulfonate, and 50 mM dithiothreitol with protease inhibitors (GE Healthcare, Piscataway, NJ), followed by sonication for 10 min at 0°C. The ultrasonicator (Sonics Model VC 750, Sonics and Materials, Newtown, CT) was set at 20% power output. After the addition of a 1% (vol/vol) nuclease mix (GE Healthcare), the cell solution was kept at room temperature for 1 h to completely solubilize the protein. The cell membranes were thoroughly broken up by re-sonication as described above. The homogenate was centrifuged for 10 min at $13,000 \times g$ at 4°C to settle the insoluble components. The supernatant fluid was obtained and its protein concentration was determined using a kit (PlusOne 2-D Quant Kit, GE Healthcare),

and aliquots (800 μ g of protein for IPEC and 1 mg of protein for mucosa) were stored at -80°C .

Comparative Proteome Analysis

Two-dimensional gel electrophoresis was run in triplicate for IPEC and in quadruplicate for mucosal samples (Wang et al., 2009c). Isoelectric focusing was performed using immobile DryStrip gels (pH 3 to 10, nonlinear, 24 cm long; GE Healthcare). Equal amounts (800 μ g of protein for IPEC cells and 1 mg of protein for mucosa) of sample were loaded onto immobile DryStrip gels strips using an in-gel sample rehydration technique. The first-dimensional isoelectric focusing was carried out at 20°C for 100,000 voltage h (IPGphor system, GE Healthcare). After isoelectric focusing, each strip was equilibrated for 15 min in 5 mL of equilibration buffer 1 (6 M urea, 1% dithiothreitol, 30% glycerol, and 50 mM Tris-Cl pH 8.8) and then in 5 mL of equilibration buffer 2 (6 M urea, 2.5% iodoacetamide, 30% glycerol, 50 mM Tris-Cl pH 8.8) for 15 min. The second dimension was performed using 12.5% SDS-PAGE gels. The gels were run at 30 mA/gel for 30 min and then at 50 mA/gel until the Bromophenol Blue came out of the gels. Analytical gels were stained with Coomassie Brilliant Blue G-250 (Amresco, Solon, OH; Wang et al., 2005). High-resolution gel images (400 dpi) were obtained using a scanner (ImageScanner, model PowerLook 2100XL, UMAX Technologies, Atlanta, GA) and image analysis was performed (Image-Master 2D Platinum Version 6.01, GE Healthcare). Differentially expressed protein spots that deviated more than 1.3-fold in their relative volume (% vol) were selected.

In-Gel Digestion and Mass-Spectrometric Analysis

Differentially expressed protein spots were cut from the gel and in-gel protein digestion was performed (Wang et al., 2005). An aliquot (3 μ L) of trypsin solution (10 μ g/mL; Amresco Inc., Solon, OH) in 25 mM ammonium bicarbonate was added to each destained gel piece after it was completely dried by vacuum centrifugation (Eppendorf Concentrator 5301, Eppendorf, Hamburg, Germany) for 30 min. The mixture was incubated at 37°C for 12 h after a 1-h period of incubation at 4°C to rehydrate it in the trypsin solution. Then, the gel pieces were vacuum-dried to evaporate the solvent, followed by the addition of 8 μ L of 5% trifluoroacetic acid (TFA). The solution was incubated at 37°C for 1 h and then transferred into a microcentrifuge tube. The gel pieces were extracted with 8 μ L of 2.5% TFA and 50% acetonitrile, and the solution was transferred to a microcentrifuge tube containing the 5% TFA extract. The third extraction was performed using 8 μ L of 100% acetonitrile and then the solution was combined with the previous 2 extracts in the microcentrifuge tube. Finally, the combined solution was dried and resolubilized

Table 1. Composition and nutrient analysis of the basal diet (as-fed basis)

Item	Content
Ingredient, %	
Corn	60.5
Skim milk powder	4.0
Whey powder	14.0
Casein	13.5
Spray-dried porcine plasma	4.5
Limestone	0.9
Dicalcium phosphate	1.3
Salt	0.3
Premix ¹	1.0
Chemical analysis ²	
CP, %	22.7
Lys, %	1.6
Ca, %	0.9
P, %	0.8
DE, MJ/kg	14.6

¹Providing the following per kilogram of complete diet: vitamin A, 10,000 IU; vitamin D₃, 1,500 IU; vitamin E, 30 IU; vitamin K₃, 2.5 mg; vitamin B₁, 1.5 mg; vitamin B₂, 10 mg; vitamin B₆, 10 mg; vitamin B₁₂, 0.05 mg; folic acid, 1 mg; biotin, 0.5 mg; niacin, 30 mg; pantothenic acid, 20 mg; Cu (copper oxide), 20 mg; Fe (iron sulfate), 100 mg; Zn (zinc oxide), 110 mg; Mn (manganous oxide), 40 mg; Se (sodium selenite), 0.3 mg; and I (ethylenediamine dihydroiodide), 0.54 mg.

²Analyzed values, except DE.

in 2 μ L of 0.5% TFA for mass-spectrometric (Bruker Reflex MALDI-TOF mass-spectrometric analysis; Bruker Daltonik, Bremen, Germany) analysis. Briefly, a saturated solution of α -cyano-4-hydroxycinnamic acid in 50% (vol/vol) acetonitrile and 0.1% (vol/vol) TFA was used as the matrix. One microliter of the matrix solution and 1 μ L of sample solution were mixed and loaded onto the Score384 target well. The variables used for the peptide mass fingerprint analysis were 20 kV accelerating voltage and 23 kV reflecting voltage. The peptide mass fingerprint analysis was performed using a search engine (MASCOT, Matrix Science, London, UK). Monoisotopic peptide masses were used to search the databases. The peptide mass accuracy was 0.2 Da. One missed cleavage was allowed. The carbamidomethylation of cysteine and oxidation of methionine were set as variable modifications (Wu et al., 2010). For protein identification by peptide mass fingerprint, peptide masses were searched against the database for other mammalian species (NCBI database, Bethesda, MD; Wang et al., 2010).

Western Blot for Analysis of Active Caspase-3

Active caspase-3 was determined by Western blot (Wang et al., 2009c). Briefly, the extracted protein sample was boiled for 5 min, electrophoresed (Bio-Rad, Richmond, CA) in 10% SDS-PAGE gel, and electroblotted (Bio-Rad) onto a polyvinylidene fluoride membrane (Millipore, Billerica, MA). After the polyvinylidene fluoride membranes were blocked with buf-

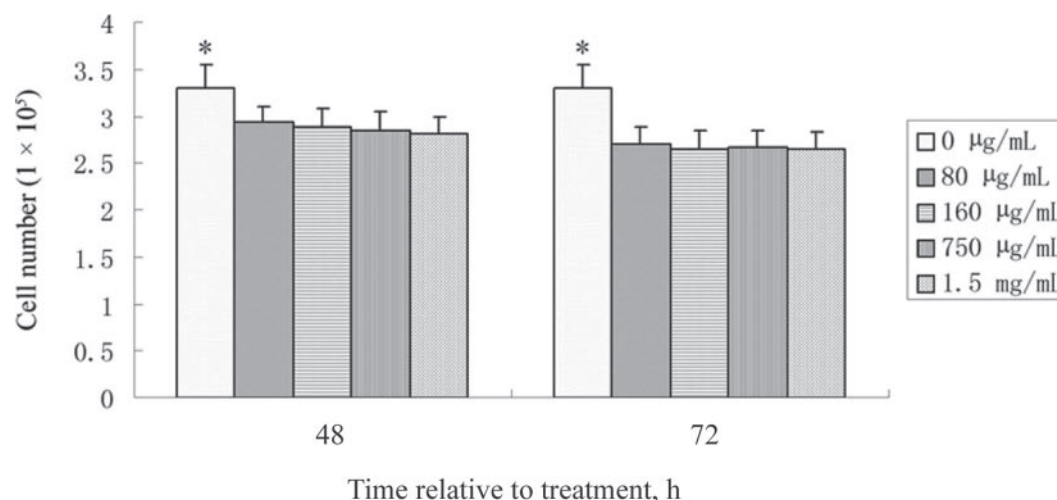


Figure 1. Effects of β -conglycinin on pig intestinal cell growth after 48 or 72 h relative to initiation of treatment with 0, 80, 160, 750, and 1,500 μ g of β -conglycinin/mL. *Differences between 0 μ g of β -conglycinin/mL vs. others (80, 160, 750, or 1,500 μ g/mL), $P < 0.05$; linear effect of β -conglycinin, $P < 0.052$.

fer containing 5% fat-free dry milk Tris-buffered saline Tween-20 (TBST; 0.05% Tween 20, 100 mmol/L of Tris-HCl, and 150 mmol/L of NaCl, pH 7.5), they were washed in TBST 4 times and incubated for 2 h with a primary polyclonal antibody (1:2,000; Sigma) for active caspase-3. The membrane was washed in the same manner as described above and incubated with horseradish peroxidase-conjugated secondary antibody (1:5,000; Sigma) for 40 min. Then, the membrane was washed 6 times for 5 min in TBST and the blots were detected using substrates (Lumi-light Western Blotting substrates, Biofuture, Beijing, China). Glyceraldehyde-3-phosphate dehydrogenase was used as the internal reference protein in this study.

Statistical Analyses

All analysis was performed using SAS (SAS Inst. Inc., Cary, NC). Differences in protein expression between control and treatment groups were determined by 1-way ANOVA with each gel as an experimental unit in proteomic comparison. The data for the cell growth experiment were analyzed using GLM procedures with each well of cells in 96 multi-well plates as an experimental unit. Linear, quadratic, and cubic polynomial contrasts were used to determine the dose-dependent effect of β -conglycinin on cell growth. Data are expressed as means $\pm$ SEM. A probability value < 0.05 was considered as statistical significance.

RESULTS

Effects of β -Conglycinin on IPEC Growth

Effects of β -conglycinin on IPEC growth are illustrated in Figure 1. After 48 and 72 h of culture with β -conglycinin at concentrations of 0, 80, 160, 750, or 1,500 μ g/mL, IPEC numbers were reduced ($P < 0.05$) in comparison with the control group, but the effect

was not dose-dependent. Particularly, cell growth did not differ as β -conglycinin concentration increased from 80 to 1,500 μ g/mL. However, when treatment time was prolonged from 48 to 72 h, IPEC exhibited a trend ($P = 0.052$) for reduced growth.

Effects of 72-h Culture with 160 μ g/mL of β -Conglycinin on IPEC Proteome

Based on our observation of the effect of β -conglycinin on IPEC growth, we treated the cells for 72 h with 160 μ g of β -conglycinin/mL, which is a concentration in the lumen of the allergic small intestine (F. Chen, unpublished data). The proteomic analyses revealed 3 upregulated protein spots (> 1.3 -fold; $P < 0.05$) and 13 downregulated protein spots (> 1.3 -fold; $P < 0.05$) in the IPEC maintained with β -conglycinin. Compared with the control group, IPEC treated with β -conglycinin exhibited a decrease in several key proteins involved in nuclei replication (e.g., heterogeneous nuclear ribonucleoprotein K isoform A isoform 14, serine hydroxymethyltransferase 1, and zinc finger protein Rlf), as well as cellular structure-related proteins (e.g., tubulin, α 1 isoform 9, and ezrin), but an increase in stress-related proteins (e.g., mitogen-activated protein kinase 8, heat shock 60 kda protein 1, and chloride intracellular channel 1). The biochemical information about these proteins is summarized in Table 2, and their appearance on the images is labeled in Figure 2.

Effects of Oral Challenge of β -Conglycinin on Jejunal Mucosal Proteome

The results of the comparative proteomic analysis between jejunal mucosae from piglets challenged with or without β -conglycinin indicated that 14 protein spots were differentially expressed. The biochemical information about these proteins is summarized in Table 3. Eleven upregulated protein spots (> 1.3 -fold; $P < 0.05$)

Table 2. Biochemical information about 16 differentially expressed spots in porcine intestinal epithelial cell (IPEC) treated with or without β-conglycinin

No. ¹	Accession ²	Name	Score ³	Sequence coverage, % ⁴	Matching (matched peptides/total) ⁵	Expression level ⁶		P-value
						Treatment	Control	
B1	gi 149410521	Angiopoietin-related protein-2	66	26	7/22	ND ⁷	388 ± 53	<0.001
B2	gi 149604613	bHLH factor Hes7	70	27	7/17	ND	241 ± 40	<0.001
B3	gi 126722635	Chloride intracellular channel 1	73	40	6/16	3,153 ± 654	1,202 ± 220	0.008
B4	gi 149410194	Enhancer of zeste homolog 2 isoform 2	60	22	12/37	ND	591 ± 84	<0.001
B5	gi 126303336	Ezrin	102	20	15/29	118 ± 27	990 ± 311	0.008
B6	gi 126324961	FYVE domain-containing dual specificity protein phosphatase FYVE-DSP1a	60	14	10/22	ND	438 ± 60	<0.001
B7	gi 194044029	Heat shock 60 kDa protein 1 (chaperonin) isoform 1	72	24	9/25	1,734 ± 410	777 ± 93	0.010
B8	gi 73946451	Heterogeneous nuclear ribonucleoprotein K isoform A isoform 14	83	25	8/27	293 ± 44	495 ± 66	0.011
B9	gi 73981556	HORMA domain containing 1	66	22	7/17	150 ± 46	686 ± 61	<0.001
B10	gi 73997866	Mitogen-activated protein kinase 8	69	21	7/14	1,763 ± 423	338 ± 58	0.004
B11	gi 194226066	Protein C9orf140	66	41	6/36	ND	291 ± 47	<0.001
B12	gi 149409104	Serine hydroxymethyltransferase 1	61	22	7/24	256 ± 69	1,185 ± 403	0.016
B13	gi 194214933	Suppression of tumorigenicity protein 18	60	13	9/23	ND	274 ± 48	<0.001
B14	gi 73971224	Valosin-containing protein transitional endoplasmic reticulum ATPase	103	26	13/24	ND	610 ± 79	0.001
B15	gi 73996547	Tubulin, α 1 isoform 9	60	31	8/32	331 ± 61	1,699 ± 456	0.006
B16	gi 73976737	Zinc finger protein Rlf	66	12	17/48	ND	280 ± 26	<0.001

¹Spot numbers are the spot protein numbers that correspond to the labels in Figure 2.

²Accession numbers are MASCOT results of matrix-assisted laser desorption/ionization time-of-flight searched from the NCBI database (<http://www.matrixscience.com>; Matrix Science, London, UK).

³Score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event. A score of ≥ 59 indicates a significant match ($P < 0.05$) with the named protein.

⁴The minimum coverage of the matched peptides in relation to the full-length sequence.

⁵The number of matched peptides in the database search relative to total peptides from the protein.

⁶Protein expression quantity, expressed as mean $\pm$ SEM, $n = 3$ gels for both β-conglycinin and IPEC treated with β-conglycinin groups. All the identified spots were differentially expressed ($P < 0.05$).

⁷No protein was detected.

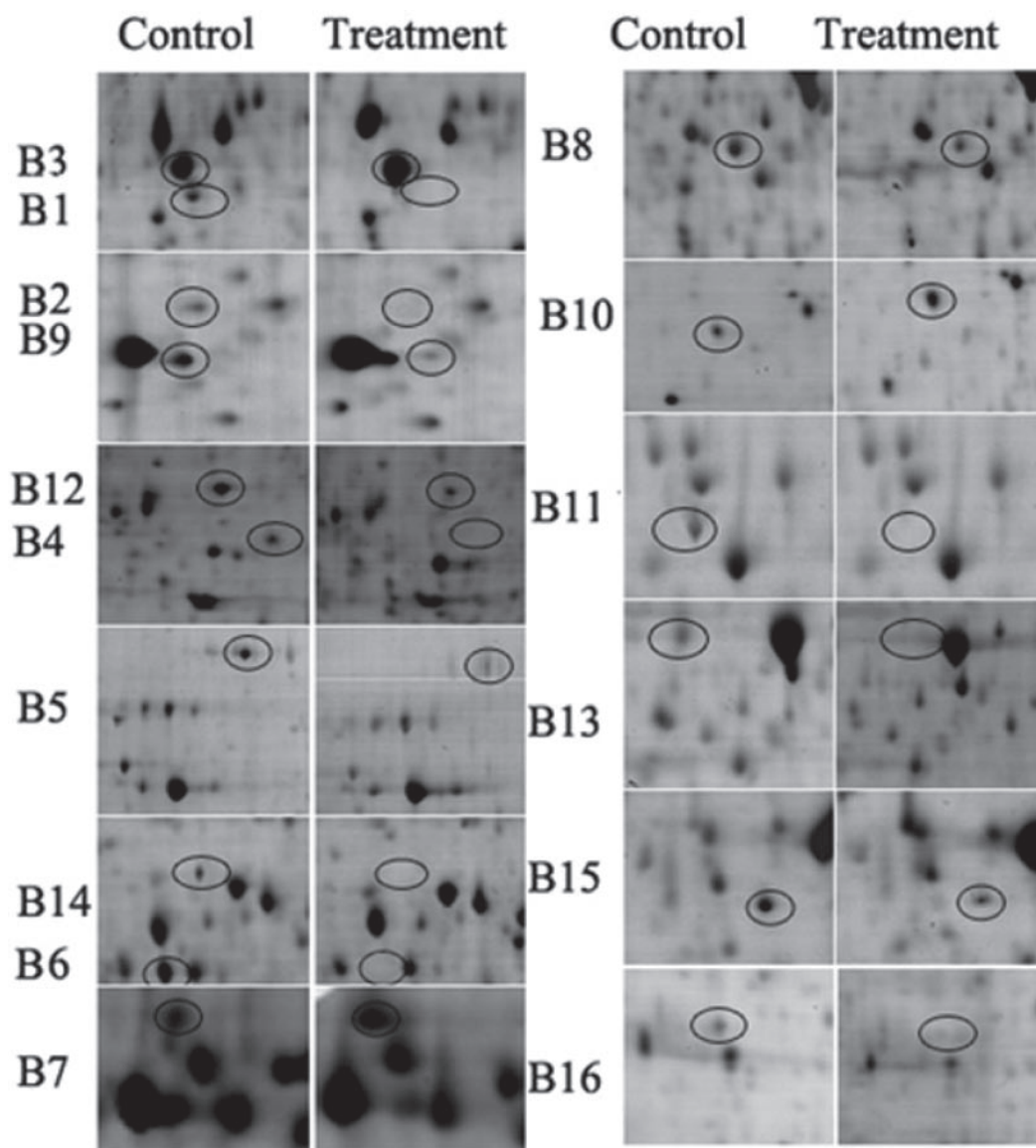


Figure 2. Abundance of differentially expressed proteins in pig intestinal cells incubated with (treatment) or without (control) β -conglycinin. B1: angiopoietin-related protein-2; B2: bHLH factor Hes7; B3: chloride intracellular channel 1; B4: enhancer of zeste homolog 2 isoform 2; B5: ezrin; B6: FYVE domain-containing dual-specificity protein phosphatase FYVE-DSP1a; B7: heat shock 60 kDa protein 1 (chaperonin) isoform 1; B8: heterogeneous nuclear ribonucleoprotein K isoform 14; B9: HORMA domain containing 1; B10: mitogen-activated protein kinase 8; B11: protein C9 or f140; B12: serine hydroxymethyltransferase 1; B13: suppression of tumorigenicity protein 18; B14: VCP transitional endoplasmic reticulum ATPase; B15: tubulin, α 1 isoform 9; and B16: zinc finger protein Rlf.

and 3 downregulated protein spots (>1.3 -fold; $P < 0.05$) were identified in the jejunal mucosa of the piglets challenged with β -conglycinin compared with the control group. The appearance of these proteins on the images is labeled in Figure 3. Challenge of β -conglycinin increased the expression of 1,4,5-triphosphate receptor-associated cyclic guanosine monophosphate kinase substrate A, peptidyl-prolyl *cis-trans* isomerase B precursor, and chloride intracellular channel protein 1, which was also upregulated in IPEC cultured with β -conglycinin. Challenge with β -conglycinin enhanced expression of valosin-containing protein (VCP) transitional endoplasmic reticulum ATPase, chaperonin containing t-complex protein 1 subunit 5 (CCT 5), chaperonin containing t-complex protein 1 subunit 8

(CCT 8), F-actin, and heat shock 70 kDa protein 8, in comparison with the control group.

Analysis of Caspase-3 Expression in IPEC and Jejunum Mucosae

Both the growth pattern of IPEC cultured with β -conglycinin and the data on in vivo jejunal mucosal proteomes revealed the increased expression of proapoptosis proteins (mitogen-activated protein kinase 8 and heat shock 60 kDa protein 1) and reduced expression of anti-apoptosis proteins (CCT 5, CCT 8, VCP transitional endoplasmic reticulum ATPase, and heat shock 70 kDa protein 8; $P < 0.05$). These results indicated that β -conglycinin induced apoptosis in vivo

Table 3. Biochemical information about 14 differentially expressed spots in jejunal mucosa of piglets treated with or without β-conglycinin

No. ¹	Accession ²	Name	Score ³	Sequence coverage, ⁴ %	Matching (matched peptides/total) ⁵	Expression level ⁶		P-value
						Treatment	Control	
D1	gi 47117646	Actin-like protein 3	209	68	29/73	259 ± 35	1,061 ± 226	0.003
D2	gi 162792	Alpha-s1-casein precursor	102	52	14/76	50 ± 12	622 ± 172	0.004
D3	gi 130493403	Cardiac ryanodine receptor	61	10	33/54	ND ⁷	340.26 ± 76	0.001
D4	gi 75812922	Chaperonin containing, t-complex protein 1, subunit 8	139	43	19/61	269 ± 14	393 ± 21	0.001
D5	gi 122140430	Chaperonin containing, t-complex protein 1, subunit 5	76	45	10/54	364 ± 150	1,060 ± 265	0.016
D6	gi 57094359	Chloride intracellular channel protein 1	78	44	8/43	466 ± 172	145 ± 44	0.035
D7	IALMV	F-actin	70	37	11/41	304 ± 70	528 ± 107	0.038
D8	gi 122139954	Heat shock 70 kDa protein 8	75	22	10/39	505 ± 39	749 ± 131	0.036
D9	gi 122143001	HSPCA protein 90	92	32	7/21	198 ± 45	363 ± 44	0.010
D10	gi 122140925	Hypothetical protein MGC127648	118	47	15/50	184 ± 22	273 ± 32	0.016
D11	gi 75050786	Inositol 1,4,5-triphosphate receptor associated cyclic guanosine monophosphate kinase substrate A	61	13	9/22	270 ± 46	137 ± 29	0.013
D12	gi 122135708	MGC137802 protein	67	35	9/39	ND	603 ± 31	0.005
D13	gi 215274238	Peptidyl-prolyl <i>cis-trans</i> isomerase B precursor	80	42	9/28	529 ± 73	253 ± 62	0.070
D14	gi 1174636	Valosin-containing protein transitional endoplasmic reticulum ATPase	108	34	22/53	191 ± 20	307 ± 65	0.040

¹Spot numbers are spot protein numbers that correspond to the labels in Figure 3.

²Accession numbers are MASCOT results of matrix-assisted laser desorption/ionization time-of-flight searched from the NCBI database (<http://www.matrixscience.com>; Matrix Science, London, UK).

³Score is $-10 \times \log(P)$, where P is the probability that the observed match is a random event. A score of ≥ 59 indicates a match ($P < 0.05$) with the named protein.

⁴The minimum coverage of the matched peptides in relation to the full-length sequence.

⁵The number of matched peptides in the database search relative to total peptides from the protein.

⁶Protein expression quantity, expressed as mean $\pm$ SEM; $n = 4$ gels for both mucosa of piglets challenged with β-conglycinin and mucosa from control groups. All the identified spots were differentially expressed ($P < 0.05$).

⁷No protein was detected.

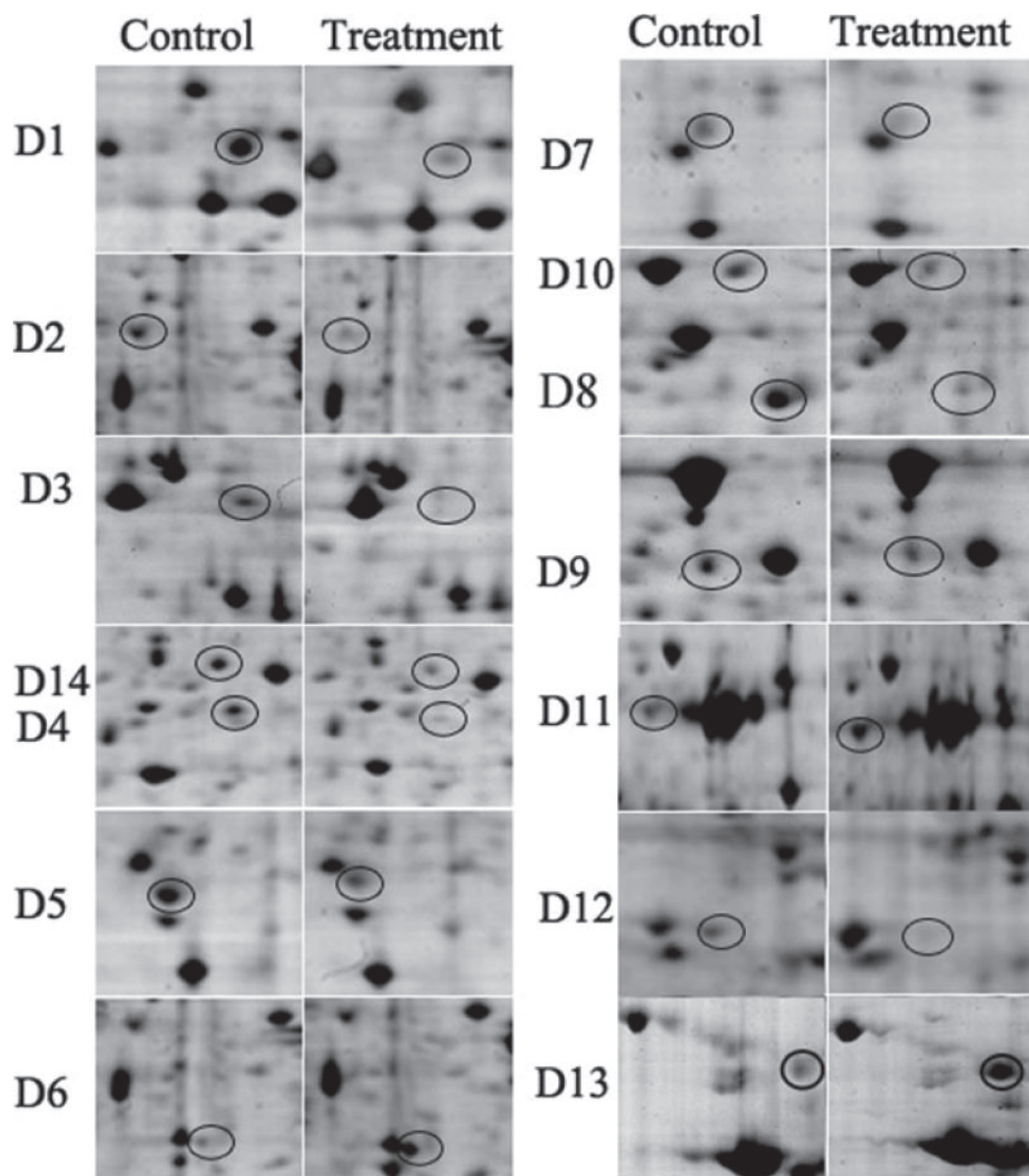


Figure 3. Abundance of differentially expressed proteins in the jejunal mucosa of piglets receiving oral administration of β -conglycinin (treatment) or vehicle solution (control). D1: actin-like protein 3; D2: α -s1-casein precursor; D3: cardiac ryanodine receptor; D4: chaperonin containing, t-complex protein 1, subunit 8; D5: chaperonin containing, t-complex protein 1, subunit 5; D6: chloride intracellular channel protein 1; D7: F-actin; D8: heat shock 70 kDa protein 8; D9: heat-shock 90-kD protein 1, α HSPCA protein 90; D10: hypothetical protein MGC127648; D11: inositol 1,4,5-triphosphate receptor associated cyclic guanosine monophosphate kinase substrate A; D12: MGC137802 protein; D13: peptidyl-prolyl *cis-trans* isomerase B precursor; and D14: valosin-containing protein transitional endoplasmic reticulum ATPase.

and in vitro. To test this hypothesis, active caspase-3 protein, a marker protein for apoptosis, was determined by Western blot (Figure 4). Our results showed that β -conglycinin supplementation increased ($P < 0.05$) the expression of active caspase-3 both in IPEC and the jejunum mucosa.

DISCUSSION

The β -conglycinin is an allergenic antigen contained in soybeans and usually induces allergies and intestinal damage in piglets and calves (L'Hocine and Boye, 2007).

Previous studies have mainly focused on the effects of allergens on immune cells and related changes, but have not examined their effects on intestinal epithelial cells. Soybean allergy results in intestinal inflammatory disorders and damage, but whether the epithelial barrier dysfunction is a consequence of inflammation or a predisposing factor remains unclear (Berin et al., 1999). Therefore, we employed the proteomic technology, as well as in vitro cell culture and in vivo administration, to test the novel hypothesis that β -conglycinin can induce changes in the intestinal proteome that can promote oxidative stress and injury in epithelial cells. Our results indicated for the first time, to our knowledge,

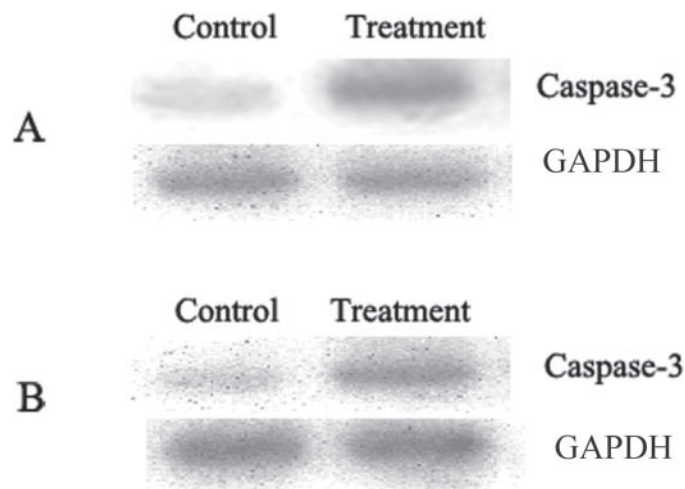


Figure 4. Western blot analysis of active caspase-3 in porcine intestinal epithelial cell (A) and the jejunal mucosa (B) in the control group (without β -conglycinin) and treatment group (with β -conglycinin), using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the reference protein.

that β -conglycinin directly inhibits intestinal epithelial-cell proliferation and stimulates expression of pro-oxidative proteins, thereby contributing to the intestinal damage observed during the allergic reaction.

Because of the lack of information in the literature regarding the doses of β -conglycinin for *in vitro* experiments, we determined the effects of β -conglycinin concentrations (0 to 1,500 $\mu\text{g/mL}$) on IPEC proliferation for 48 and 72 h. A novel and important finding of the current study is that β -conglycinin was a potent inhibitor of IPEC growth. After 48 or 72 h of culture with this allergen, IPEC numbers were substantially reduced in the treatment group in comparison with the control, and a plateau effect was observed at 80 $\mu\text{g/mL}$ of β -conglycinin. As a protein, β -conglycinin added into the cell medium may mimic its presence in the lumen of the intestine, and could be degraded to AA and peptides by enzymes secreted from IPEC (Wang et al., 2009b). However, some intact proteins may escape digestion, inhibit cell proliferation, and alter extracellular osmolarity. The balance of these 2 opposite actions determines the growth pattern of IPEC. The suppressed growth of β -conglycinin-treated IPEC might result from an effect of this allergen as an inducer of stress.

As an initial step to understand how β -conglycinin affects the growth of intestinal cells, we examined protein expression in IPEC *in vitro* and the jejunal mucosa of piglets *in vivo*. A novel finding of this study is that β -conglycinin decreased the expression of serine hydroxymethyltransferase 1, which plays an important role in glycine synthesis and 1-carbon-unit metabolism (Wu, 2009). Previous studies showed that serine hydroxymethyltransferase 1 activity was increased several-fold during cell proliferation to meet the need for *de novo* nucleotide biosynthesis (Eichler et al., 1981). The decreased expression of serine hydroxymethyltrans-

ferase 1 in β -conglycinin-treated cells likely contributed to impairment of cell kinetics and growth. On the other hand, mitogen-activated protein kinase 8, which is critical for signal transduction in the immune response and mediates numerous physiological processes, including proliferation, differentiation, and apoptosis (Rao, 2001; Dong et al., 2002), exhibited greater expression in β -conglycinin-treated cells. It has been reported that stressors, toxins, and receptor agonists activate mitogen-activated protein kinase 8, which drives both apoptosis and expression of related genes to induce inflammation (Widmann et al., 1999; Hassoun and Wang, 2000). The greater expression of mitogen-activated protein kinase 8 indicates that IPEC might experience severe stress in response to β -conglycinin. This supposition is consistent with the increased expression of heat shock 60 kDa protein 1, which is a marker protein for cell stress (Kuwabara et al., 1993).

Ezrin, the membrane cytoskeleton cross-linker, is a component of actin-containing cell surface structures in a wide variety of cells and plays an important role in cell morphology, cytoskeleton reorganization, adhesion, invasion, and metastasis (Bonilha, 2007). Also, identified as an essential component of the brush border microvilli that covers the apical surface of polarized epithelial cells, ezrin contributes to the linkage between the lateral membrane and actin core for microvillus formation. Therefore, ezrin is required for proper apical membrane assembly (Berryman et al., 1995; Speck et al., 2003). Ezrin has also been implicated in organization of the apical terminal web region, from which the microvilli project (Mathan et al., 1976). In the absence of ezrin, the underlying web region becomes disorganized and the microvilli are projected in a disorderly manner from its surface, apical junctions loosen, and the microvilli disappear (Saotome et al., 2004). Investigations also reported that a deficiency of ezrin in the secretory canaliculus of the gastric wall can impair HCl secretion, indicating a vital role for ezrin in vesicle transport (Tamura et al., 2005). Therefore, the down-regulation of ezrin in β -conglycinin-treated intestinal cells can result in abnormal morphology and dysfunction of cells.

Another interesting and novel observation from the current study is that addition of β -conglycinin both *in vivo* and *in vitro* increased expression of chloride intracellular channel 1, but decreased the expression of cytoskeletal proteins (tubulin, F-actin, actin-like protein 3). Cytoskeleton, identified as a key participant in signal transduction and other regulatory pathways within eukaryotic cells, is involved in nearly all aspects of cell behavior, such as cell motility, shape, and cytokinesis (Pearce-Pratt et al., 1994). Given its function as an efficient sensor or a mediator of regulation of various ion transporters and channels, cytoskeleton would be an important target for many intracellular or extracellular stimuli. In support of this notion, Ares et al. (2005) reported that PTX-6, a biotoxin produced by harmful

marine microalgae, exerted potent cytotoxic effects on enterocytes with a large reduction of F-actin microfilaments. In cells, F-actin forms a continuous ring beneath the membrane. This rigid meshwork protects the cell from the effect of stimuli on the cytoskeleton. In the present study, the β -conglycinin-induced reduction in F-actin, tubulin, and actin-like protein-3, which are all known to play important roles in cytoskeleton structure and function, may weaken cellular shield and contribute to abnormal morphological changes in pig intestinal cells. This finding indicates that the damaged intestinal villous observed in fetuses and young animals with a soybean allergy likely results from abnormal changes of cytoskeleton-related proteins, as recently reported for the injured intestine in soy protein-fed goats (Kuhla et al., 2007). Because there is a close link between structure and function, upregulation of chloride intracellular channel 1 observed in β -conglycinin-treated cells is also consistent with the report that chloride intracellular channel 1 was positively regulated by the disruption of F-actin (Singh et al., 2007).

All those findings on reduced cell proliferation, damaged cytoskeleton, and impaired function indicate that β -conglycinin may induce apoptosis of intestinal cells both in vivo and in vitro. This conclusion is supported by the increased expression of activated caspase-3, a marker protein in the apoptotic process (Haynes et al., 2009; Wang et al., 2009c). Initiation of apoptosis depends on the balance of pro-apoptosis and anti-apoptosis. Both mitogen-activated protein kinase 8 and heat shock 60 kDa protein 1, which are considered to promote pro-apoptosis, were upregulated, whereas CCT5, CCT 8, VCP transitional endoplasmic reticulum ATPase, and heat shock 70 kDa protein 8, all of which are anti-apoptosis proteins, were downregulated when β -conglycinin was added to IPEC or orally administered to young pigs. Under the influence of stress, the apoptosis process was started and the caspase pathway was activated (Ruemmele et al., 2002). As a result, cell morphology and metabolism underwent a series of dramatic changes, which were characterized by inhibition of the synthesis of cytoskeletal protein and enhanced degradation of actin (Steller, 1995; Guénal et al., 1997). Furthermore, contact between intestinal cells is reduced and the extracellular matrix is compromised due to decreased expression of ezrin (Frisch and Francis, 1994), leading to damage and impaired growth of intestinal cells.

In conclusion, in contrast to the previous view that intestinal damage was exclusively provoked by immediate allergic reactions and a delayed allergy, we found that β -conglycinin altered cytoskeleton, function, and replication of pig intestinal cells, all of which indicate that the allergen could directly affect the small intestine. Our results provide new insight into the mechanisms responsible for allergic damage of the small intestine in piglets and other neonates consuming β -conglycinin-containing foods.

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