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β-Conglycinin-Induced Intestinal Porcine Epithelial Cell Damage via the Nuclear Factor κB/Mitogen-Activated Protein Kinase Signaling Pathway

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ABSTRACT: Soybean allergy is a serious health risk to humans and animals; β-conglycinin is the primary antigenic protein in soybean. Intestinal porcine epithelial (IPEC-J2) cells were used as an *in vitro* physiological model of the intestinal epithelium to study the effects of different concentrations of soybean antigen protein β-conglycinin to identify the involved signaling pathways. The cells were divided into eight groups and either untreated or treated with different concentrations of β-conglycinin, pyrrolidine dithiocarbamate (PDTC), N_{ω}-nitro-L-arginine methyl ester hydrochloride (L-NAME), SP600125, and SB202190 either alone or in combination. The cells were incubated with 1, 5, and 10 mg·mL^{-1} β-conglycinin or 5 mg·mL^{-1} β-conglycinin and 1 μmol·L^{-1} nuclear factor κB (NF-κB) inhibitor (PDTC), inducible nitric oxide synthase inhibitor (L-NAME), c-Jun N-terminal kinase (JNK) inhibitor (SP600125), and p38 inhibitor (SB202190) for 24 h, separately; controls were left untreated. The mRNA, protein, and phosphorylation levels of NF-κB, p38, and JNK were higher in the treated groups than in the control group. β-Conglycinin decreased tight junction distribution, destroyed the cytoskeleton of IPEC-J2 cells, and caused cell death. After the addition of the inhibitors, β-conglycinin-induced IPEC-J2 cell damage was significantly reduced. β-Conglycinin caused damage to IPEC-J2 cells via the mitogen-activated protein kinase/NF-κB signaling pathway. The results of this study are crucial for exploring the mechanisms underlying allergic reactions caused by soybean antigen proteins.

KEYWORDS: *β-conglycinin, allergenic reactions, IPEC-J2, MAPK, NF-κB*

INTRODUCTION

Soybean is nutritionally valuable because of its balanced amino acid profile and high protein content.[1] Soybean is present in many pet foods but is a commonly allergenic food source for both humans[2] and animals,[3] requiring food allergen labeling. Previous studies have found that piglets are particularly susceptible to soybean allergy, and transient hypersensitivity reactions often arise when weaned onto a soybean antigen protein-containing diet.[4−6] Therefore, it is essential to attain a deeper understanding of soybean allergy. 11S glycinin (Gly m 6) and 7S β-conglycinin (Gly m 5) are the most abundant seed storage proteins in soybean, and they are both significant allergens.[1] As a glycoprotein, β-conglycinin consists of three subunits: α′ (∼72 kDa), α (∼68 kDa), and β (∼50 kDa). The structural homology between these three subunits is relatively high, about 70−75%, and these three subunits have been identified as allergens.[7] The epitopes of the β subunit of the soybean seed storage protein β-conglycinin were shown to be antigenic in piglets, dogs, fish, and rabbits, suggesting possible cross-species reactivity. Positive skin tests to soybean meal have been observed in equines and piglets, indicating a potential role for soybean in chronic obstructive pulmonary disease.[8] Additionally, in BALB/c mice, the shaved epidermis on the back was exposed to the crude soybean extract and it was found that immunoglobulin E (IgE) specifically and preferentially binds to the 7S globulin β subunit.[9]

β-Conglycinin inhibited the growth of intestinal cells, destroyed the cytoskeleton, and induced gastrointestinal damage, ultimately leading to apoptosis of intestinal cells in piglets.[10] The intestinal tract, as a barrier between the host and external environment, responds to prevent the entrance of harmful entities, including bacteria, endotoxin, toxic macromolecules, and luminal antigens.[11] Intestinal porcine epithelial (IPEC-J2) cells were separated from the small intestine of pigs; mainly, non-transformed IPEC-J2 retaining most of its epithelial properties is the best porcine epithelial cell culture model.[12] Tight junctions (TJs) play a vital role in maintaining the mucosal barrier function and intestinal health of animals and humans, consisting of claudins, occludin, and other transmembrane proteins. These proteins are aggregated and stabilized by the zonula occludens (ZOs) and cytoskeleton proteins.[13] Claudins and occludin are located at the top and base of the lateral membrane, respectively.[14] A previous study estimated the effect of β-conglycinin on intestinal epithelial permeability and integrity, TJ distribution, and TJ protein expression and illustrated a dose-dependent decrease in the expression of TJ proteins with increasing β-conglycinin levels.[15] Piglets are susceptible to allergies caused by soybeans. Thus, the presence of allergenic ingredients, including β-conglycinin products and their enzyme-hydrolyzed peptides, extremely limits soybean products consumed by piglets. Soybean allergies typically induce intestinal inflammatory diseases, characterized by atrophy and proliferation of villi in the crypt

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Table 1. Primer Sequences for qRT-PCR Amplification

	accession number	primer	sequence (5′ → 3′)	product (bp)
p38	XM_001929490.5	forward	AAGACTCGTTGGAACCCCAG	150
		reverse	TCCAGCAAGTCAACAGCCAA	
JNK	NC_010456.5	forward	CAAGCACCTTCACTCTGCTG	168
		reverse	TCAGGTGCTCTGTAGTAGCG	
iNOS	NM_001143690.1	forward	GGGTCAGAGCTACCATCCTC	114
		reverse	CGTCCATGCAGAGAACCTTG	
IKKα	NM_001114279.1	forward	CACTCTTACAGCGACAGCAC	145
		reverse	CCACCTTGGGCAGTAGATCA	
IKKβ	NM_001099935.1	forward	ACCTGGCTCCCAACGACTT	184
		reverse	AGATCCCGATGGATGATTCTG	
COX-2	NC_000845.1	forward	TGCACGGCGGCAATATTAAA	156
		reverse	AGTGGAAGTGTGCGACTACA	
NF-κB p65	100514505	forward	CTCGCTCGCACTCTTGCTCTC	173
		reverse	AATAGGCGGACGAGGCAGAAG	
ZO-1	NC_010443.5	forward	GGGCTCTTGGCTTGCTATTC	160
		reverse	AAGGCCTCGGAATCTCCAAA	
claudin-1	NC_010455.5	forward	GGCAGATCCAGTGCAAAGTC	94
		reverse	CCCAGCAGGATGCCAATTAC	
occludin	NC_010458.4	forward	CATTATGCACCCAGCAACGA	168
		reverse	GCACATCACGATAACGAGCA	
AP-1	NC_010448.4	forward	GGTGGCCCAGCTTAAACAAA	72
		reverse	AACTGCTGCGTTAGCATGAG	
CREB	NC_010457.5	forward	TTGCCACATTAGCCCAGGTA	67
		reverse	AAAGTTACGGTGGGAGCAGA	
actin	414396	forward	CTGGACTTCGAGCAGGAGATGG	168
		reverse	TTCGTGGATGCCGCAGGATTC	

Table 2. Antibodies and Dilutions for Western Blotting

antibody	supplier	dilution
NF-κB	Proteintech 10745-1-AP	1:1000
p-NF-κB	Santa Cruz sc-136548	1:1000
iNOS	Novusbio NBP1-33780	1:1000
IKKα/β	Immunoway YT2301	1:1000
p-IKKα/β	Affbiotech AF3013	1:1000
JNK	Santa Cruz sc-7345	1:500
p-JNK	ImmunoWay YP0157	1:500
p38	Immunoway YT3513	1:800
p-p38	Santa Cruz sc-166182	1:800
ERK	Santa Cruz sc-514302	1:1000
p-ERK	Santa Cruz sc-81492	1:1000
c-Jun	Bioss bs-0670R	1:1500
p-c-Jun	Affbiotech AF3095	1:1000
c-Fos	Abcam ab190289	1:1000
p-c-Fos	Affbiotech AF0132	1:1000
claudin-1	Novusbio NBP1-77036	1:1000
ZO-1	Novusbio NBP1-85047	1:1000
occludin	Abcam ab31721	1:1000
β-actin	Absin abs132001	1:1000

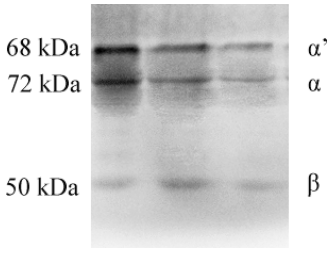

Figure 1. SDS–PAGE for preparation of β-conglycinin.

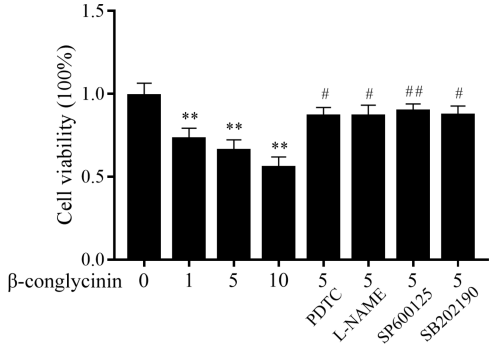

Figure 2. Cell viability of IPEC-J2 cells after incubation with β-conglycinin or inhibitors measured by the CCK-8 assay. The experiments were repeated in triplicate, and each bar represents the mean ± SD. (∗) $p < 0.05$ shows a highly significant difference compared to the control group. (∗∗) $p < 0.01$ shows a highly significant difference compared to the control group. (#) $p < 0.05$ shows a highly significant difference compared to the 5 mg·mL^{-1} β-conglycinin group. (##) $p < 0.01$ shows a highly significant difference compared to the 5 mg·mL^{-1} β-conglycinin group.

and accelerated intestinal cell proliferation, apoptosis, and migration.[16,17] However, few studies have evaluated the effects of β-conglycinin on signaling pathway mechanisms.

Nuclear factor κB (NF-κB) is an essential transcriptional factor that plays pivotal roles in inflammatory responses by regulating genes encoding pro-inflammatory proteins, such as inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2). Other than NF-κB, the activator protein (AP)-1 complex, composed of c-Fos and c-Jun subunits, regulates genes that encode inflammatory mediators.[18] Extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK) are three

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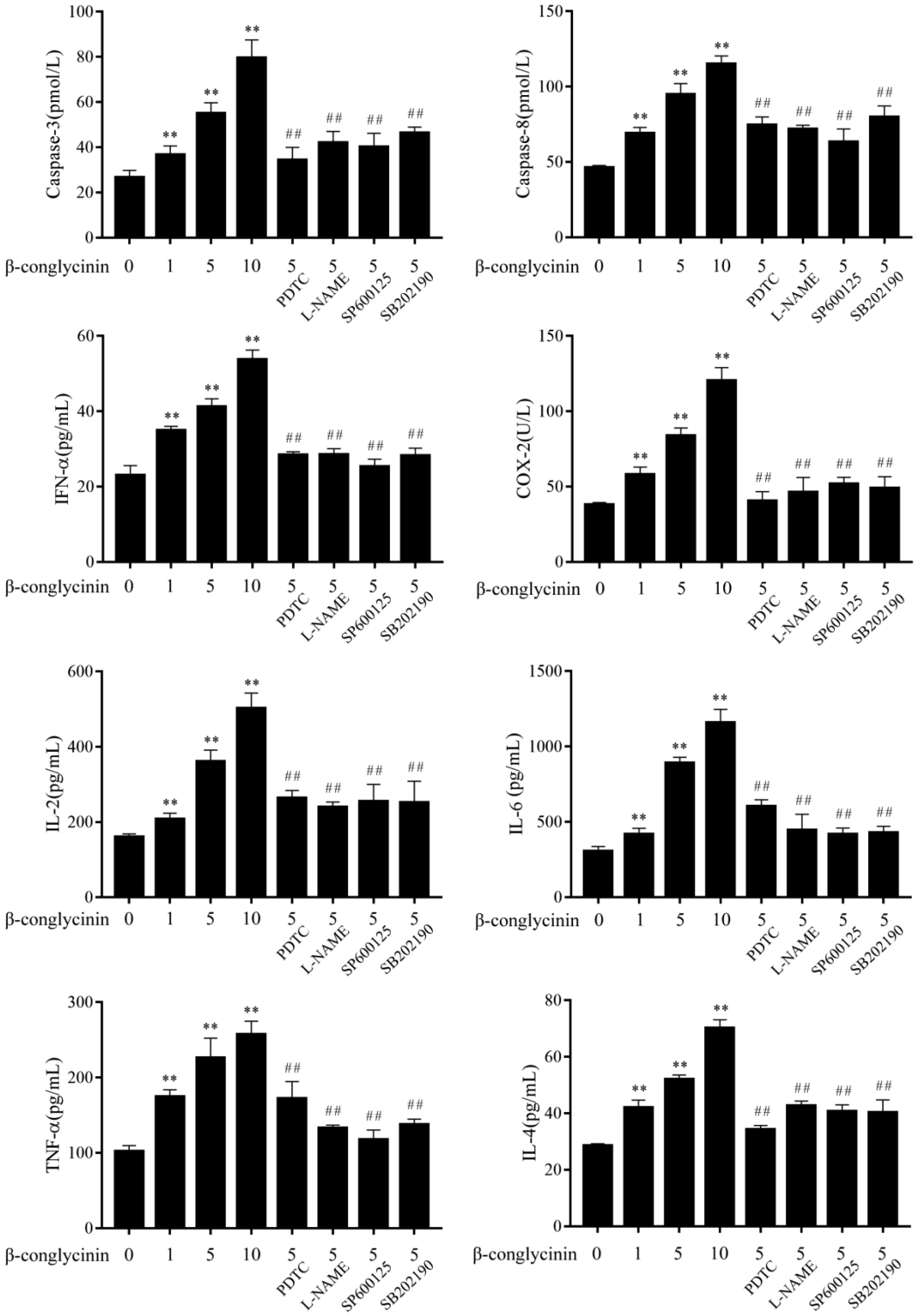

Figure 3. Cytokine, caspase-3/-8, and COX-2 expression levels of IPEC-J2 cells after incubation with β-conglycinin or inhibitors measured by ELISA. The experiments were repeated in triplicate, and each bar represents the mean ± SD. (∗) $p < 0.05$ shows a highly significant difference compared to the control group. (∗∗) $p < 0.01$ shows a highly significant difference compared to the control group. (#) $p < 0.05$ shows a highly significant difference compared to the 5 mg·mL^{-1} β-conglycinin group. (##) $p < 0.01$ shows a highly significant difference compared to the 5 mg·mL^{-1} β-conglycinin group.

downstream pathways of the mitogen-activated protein kinase (MAPK) pathways; MAPK regulates cell proliferation and immune reactions in the intestine.[19] High levels of iNOS and tumor necrosis factor α (TNF-α) could combine to receptors on the cellular membrane and ultimately activate NF-κB.[20] NF-κB is essential for the immune system and modulation of cytokine expression and effector enzymes as well as binding to various receptors involved in immunization.[21]

The intestinal epithelium not only absorbs dietary factors but also restricts the permeation of toxic substances. Typically,

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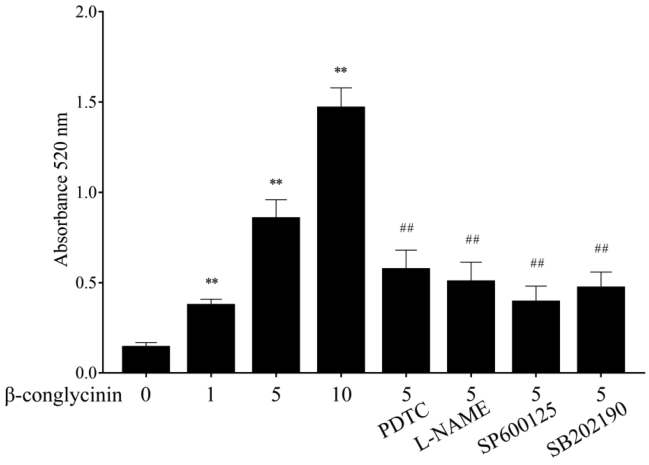

Figure 4. Alkaline phosphatase activity of IPEC-J2 cells after incubation with β-conglycinin or inhibitors. The experiments were repeated in triplicate, and each bar represents the mean ± SD. (*) $p < 0.05$ shows a highly significant difference compared to the control group. (**) $p < 0.01$ shows a highly significant difference compared to the control group. (#) $p < 0.05$ shows a highly significant difference compared to the 5 mg·mL^{-1} β-conglycinin group. (##) $p < 0.01$ shows a highly significant difference compared to the 5 mg·mL^{-1} β-conglycinin group.

most dietary proteins are digested to produce small peptides or amino acids that are then absorbed into intestinal epithelial cells. Small amounts of intact allergic protein may be endocytosed into intestinal epithelial cells to degrade and lose its antigenicity.[22,23] Moreover, the ability of allergens to go through the epithelial barrier could be determined by the permeability of TJs or their immunogenic activity.[24,25] Previous research on food allergens using *in vitro* models has focused on their effects on TJs. Price et al. indicated that the allergens in peanut can affect the intestinal barrier permeability and TJ localization in Caco-2 cells, which cross the epithelial monolayer by the paracellular pathway.[26] For instance, Zhao et al. illustrated that incubation with β-conglycinin from soybean induced the downregulation of TJ proteins in IPEC-J2 cells.[15] Therefore, in this study, IPEC-J2 cells are employed as a intestinal epithelium model to study the effects of β-conglycinin on cells and to identify β-conglycinin-related signaling pathways.

The target of the present study was to investigate β-conglycinin-induced IPEC-J2 cell damage via the NF-κB/MAPK signaling pathway. We used IPEC-J2 cells to mimic *in vivo* conditions closely.

MATERIALS AND METHODS

Preparation of β-Conglycinin. β-Conglycinin was kindly provided by Professor Shuntang Guo of China Agricultural University (patent number 200 410 029 589.4, China). We used crude soy protein to purify β-conglycinin by alkaline saponification, pI precipitation, and gel filtration as described previously.[27] The protein contents were determined after lyophilization. It was performed at 80 V through the stacking gel and 120 V through the separation gel, and then the gel was stained with Coomassie Brilliant Blue G250 (Servicebio, Wuhan, China) at 26 °C for 30 min. The gel was scanned by Imagine Lab System with SYSTEM GelDoc XR$^+$ (Bio-Rad, Hercules, CA, U.S.A.), and the purity of β-conglycinin was measured by Quantity One analysis software (Bio-Rad, Hercules, CA, U.S.A.) according to Hao et al.[4]

Culture Conditions. The IPEC-J2 cell line used in this study was provided by the China Center for Type Culture Collection (Wuhan, China) and cultured with RPMI1640 medium (Thermo Fisher Scientific, Waltham, MA, U.S.A.), adding 10% fetal bovine serum (FBS, Clark Bioscience, Richmond, VA, U.S.A.), 1% penicillin, and 1% streptomycin under 37 °C with 5% CO_2 in a humidified atmosphere. The medium was changed every 2 days to avoid nutrient depletion.

Experimental Methods and Sample Collection. IPEC-J2 cells were seeded into different well plates (BD Falcon, Corning, Inc., Corning, NY, U.S.A.); after a 24 h stabilization period, fresh, untreated cells were defined as the control group and incubated with 1, 5, or 10 mg·mL^{-1} β-conglycinin or 5 mg·mL^{-1} β-conglycinin and 1 μmol·L^{-1} pyrrolidine dithiocarbamate (PDTC), N_ω-nitro-L-arginine methyl ester hydrochloride (L-NAME, Beyotime Biotechnology, Shanghai, China), SP600125, or SB202190 (MedChem Express, Shanghai, China) for 24 h. The inhibitor group was pretreated with 1 μmol·L^{-1} PDTC, L-NAME, SP600125, and SB202190 for 30 min.

Cell Viability Assay. IPEC-J2 cells were seeded into 96-well plates at a density of 2 × 10^3 cells/well and incubated with 1, 5, and 10 mg·mL^{-1} β-conglycinin or 5 mg·mL^{-1} β-conglycinin and 1 μmol·L^{-1}

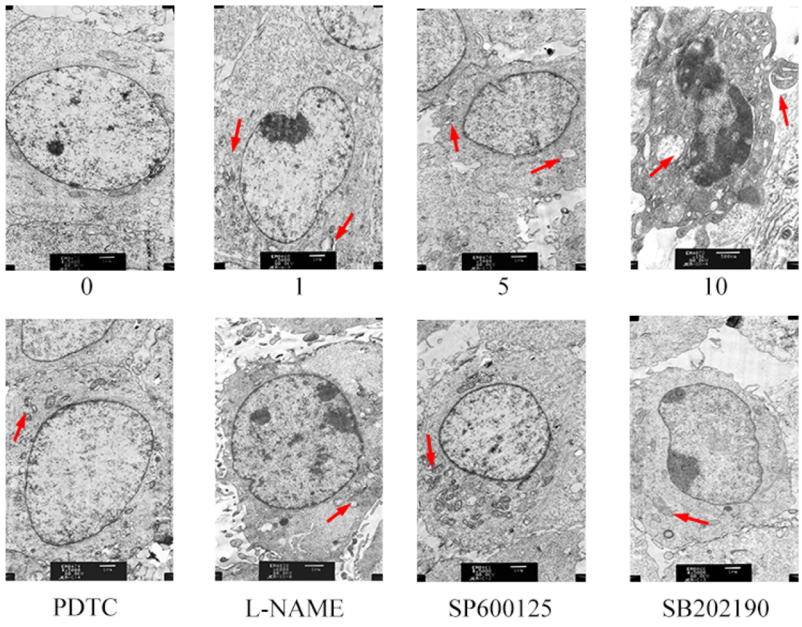

Figure 5. Electron microscopic observations of IPEC-J2 cells after incubation with β-conglycinin or inhibitors (5000×, 80.0 kV).

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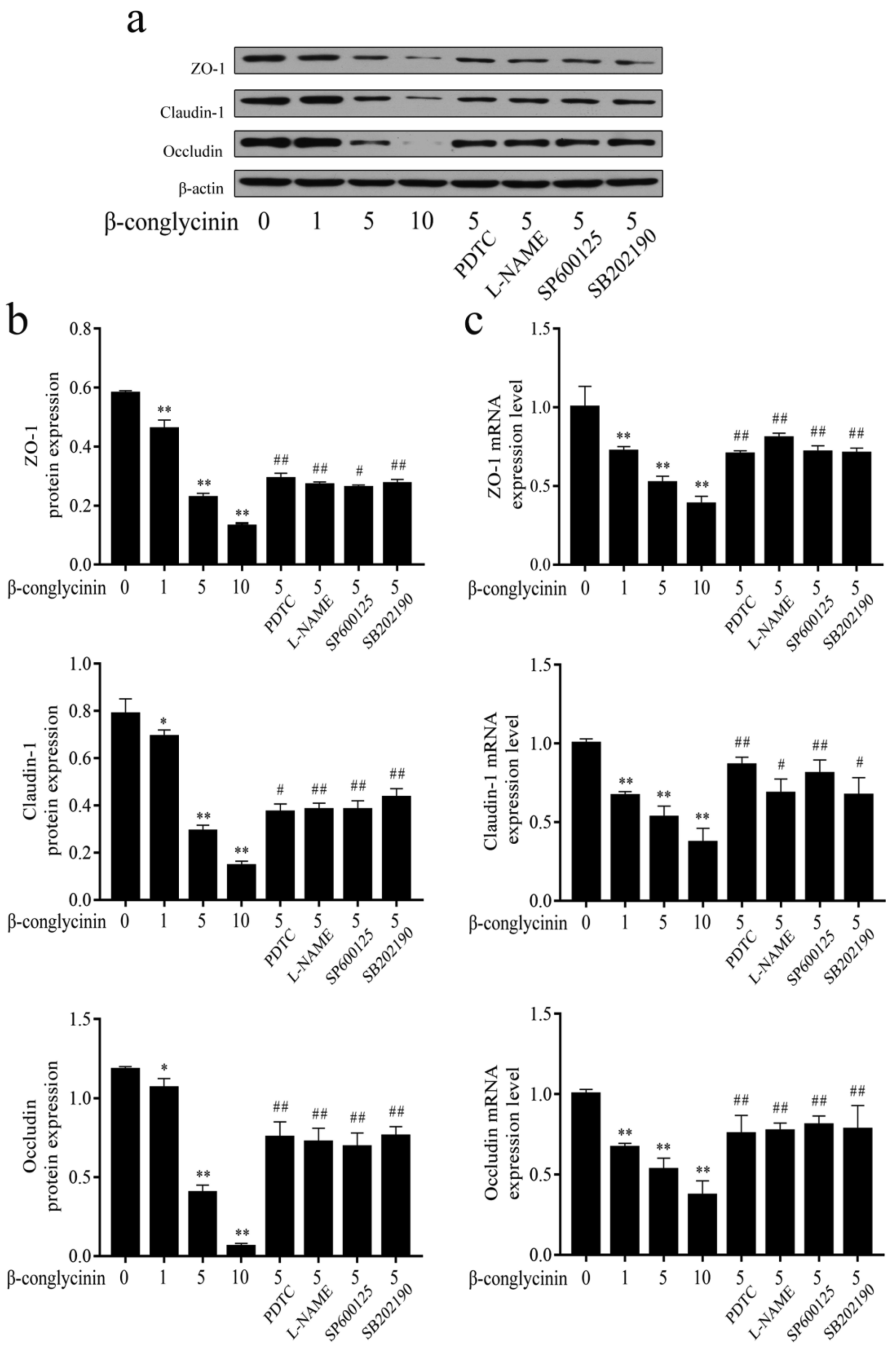

Figure 6. Effects of ZO-1, claudin-1, and occludin of IPEC-J2 cells after incubation with β-conglycinin or inhibitors. (a) Western blotting of ZO-1, claudin-1, and occludin of IPEC-J2 cells after incubation with β-conglycinin or inhibitors. (b) Western blotting and qRT-PCR of ZO-1, claudin-1, and occludin of IPEC-J2 cells after incubation with β-conglycinin or inhibitors. (c) Western blotting and qRT-PCR of ZO-1, claudin-1, and occludin of IPEC-J2 cells after incubation with β-conglycinin or inhibitors. The experiments were repeated in triplicate, and each bar represents the mean ± SD. (*) $p < 0.05$ shows a highly significant difference compared to the control group. (**) $p < 0.01$ shows a highly significant difference compared to the control group. (#) $p < 0.05$ shows a highly significant difference compared to the 5 mg·mL^{-1} β-conglycinin group. (##) $p < 0.01$ shows a highly significant difference compared to the 5 mg·mL^{-1} β-conglycinin group.

PDTC, L-NAME, SP600125, and SB202190 for 24 h, separately. After culturing for 24 h, 10 μL of CCK-8 (Dojindo Molecular Technologies, Kumamoto, Japan) reagent was added and incubated for 1 h under 37 °C. Optical density (OD) values were detected at 450 nm by a Multiskan MS plate reader (Thermo Fisher Scientific, Waltham, MA, U.S.A.).

Enzyme-Linked Immunosorbent Assay (ELISA). A volume of 3 × 10^5 cells was transferred to a 6-well plate and cultured for 24 h. Next, 500 μL of 0.1 mol·L Tris−HCl (pH 7.4, Beyotime Biotechnology, Shanghai, China) containing 0.1% Triton X-100 (Beyotime Biotechnology, Shanghai, China) was added to each sample. The sample was placed in ice water for sonication; and the cell lysate was centrifuged at 1000g for 10 min; and the supernatant was taken. Caspase-3/-8, TNF-α, interferon α (IFN-α), COX-2, and interleukin (IL)-2/-6/-4 levels were evaluated after adding β-conglycinin, PDTC, L-NAME, SP600125, or SB202190 to the IPEC-J2 cells according to the instructions. The concentrations were estimated on the basis of a standard curve.

Alkaline Phosphatase Activity. Cell membrane integrity was evaluated by measuring alkaline phosphatase activity in the IPEC-J2

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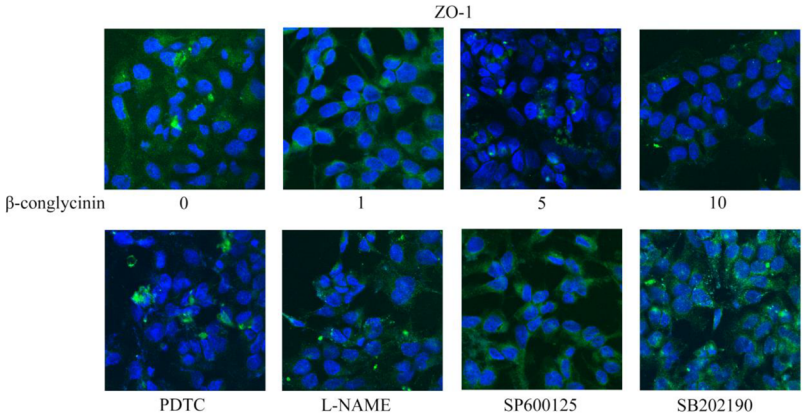

Figure 7. Detection of ZO-1 protein expression in IPEC-J2 cells by immunofluorescence after incubation with β-conglycinin or inhibitors. Immunofluorescence staining observations were taken with a laser scanning confocal microscope (magnification of 800×).

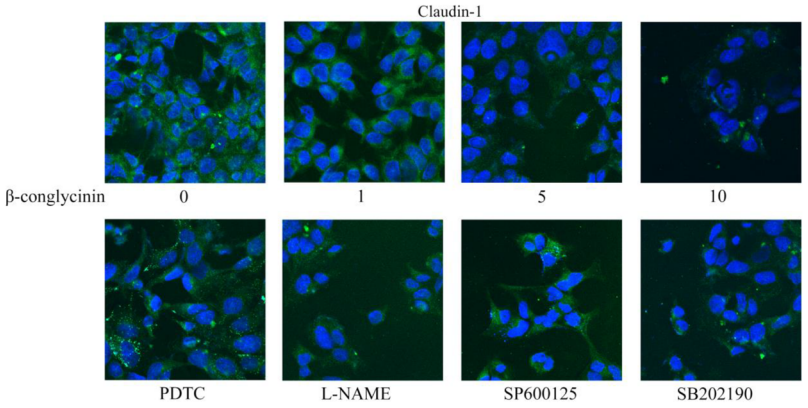

Figure 8. Detection of claudin-1 protein expression in IPEC-J2 cells by immunofluorescence after incubation of β-conglycinin or inhibitors. Immunofluorescence staining observations were taken with a laser scanning confocal microscope (magnification of 800×).

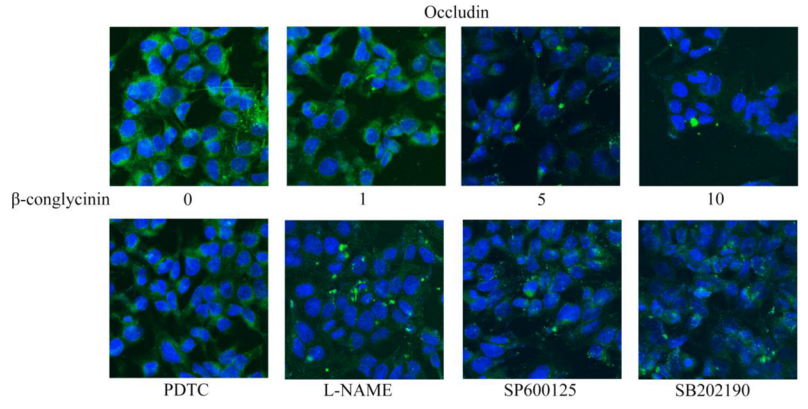

Figure 9. Detection of occludin protein expression in IPEC-J2 cells by immunofluorescence after incubation with β-conglycinin or inhibitors. Immunofluorescence staining observations were taken with a laser scanning confocal microscope (magnification of 800×).

culture supernatant. Cells were seeded in a 96-well microplate, treated with 1, 5, or 10 mg·mL^{-1} β-conglycinin or 5 mg·mL^{-1} β-conglycinin and PDTC, L-NAME, SP600125, or SB202190 for 24 h. Alkaline phosphatase activity in the culture supernatant was detected by an alkaline phosphatase test kit (Jiancheng, Nanjing, China), according to the instructions of the manufacturer.

Transmission Electron Microscopy (TEM). After treatment, the cells were post-fixed in phosphate-buffered 4% glutaraldehyde for 12 h under 4 °C, washed 3 times in phosphate buffer (5000*g* for 15 min at 4 °C), and fixed in 1% osmic acid for 2 h. Subsequently, samples were washed 3 times in phosphate buffer under 4 °C, dehydrated for 30 min in a graded alcohol series (approximately 30−90%), and further dehydrated for 30 min in graded acetone (90 and 100%). Ultrathin sections were cut and stained; digital images were taken with JEM-1230 TEM (JEOL, Akishima, Tokyo, Japan), as depicted by Wang et al.[28]

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). The IPEC-J2 cells were grown at a density of 1.3 × 10^6 cells/well in 24-well plates and used for the RNA extraction. Total RNA from IPEC-J2 cells was extracted using RNAiso Plus (TaKaRa Biotechnology, Shiga, Japan). Next, 200 μL of chloroform was added to the sample, which was shaken 20 times and centrifuged (12000*g* for 15 min). The resulting supernatant (500 μL) was mixed with 500 μL of isopropanol

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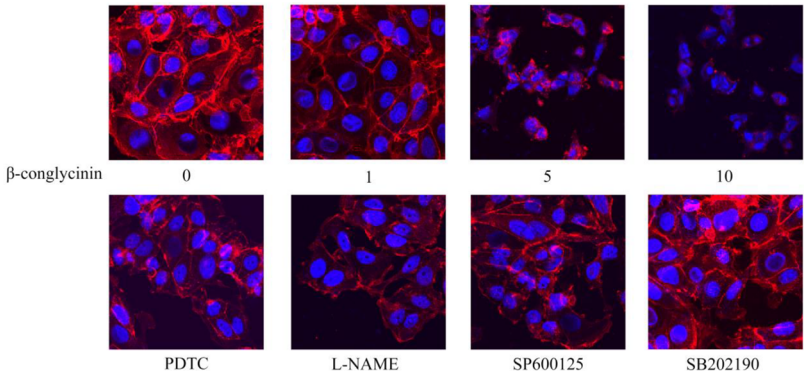

Figure 10. Detection of the cytoskeleton of IPEC-J2 cells by immunofluorescence after incubation with β-conglycinin or inhibitors. Immunofluorescence staining observations were taken with a laser scanning confocal microscope (magnification of 800×).

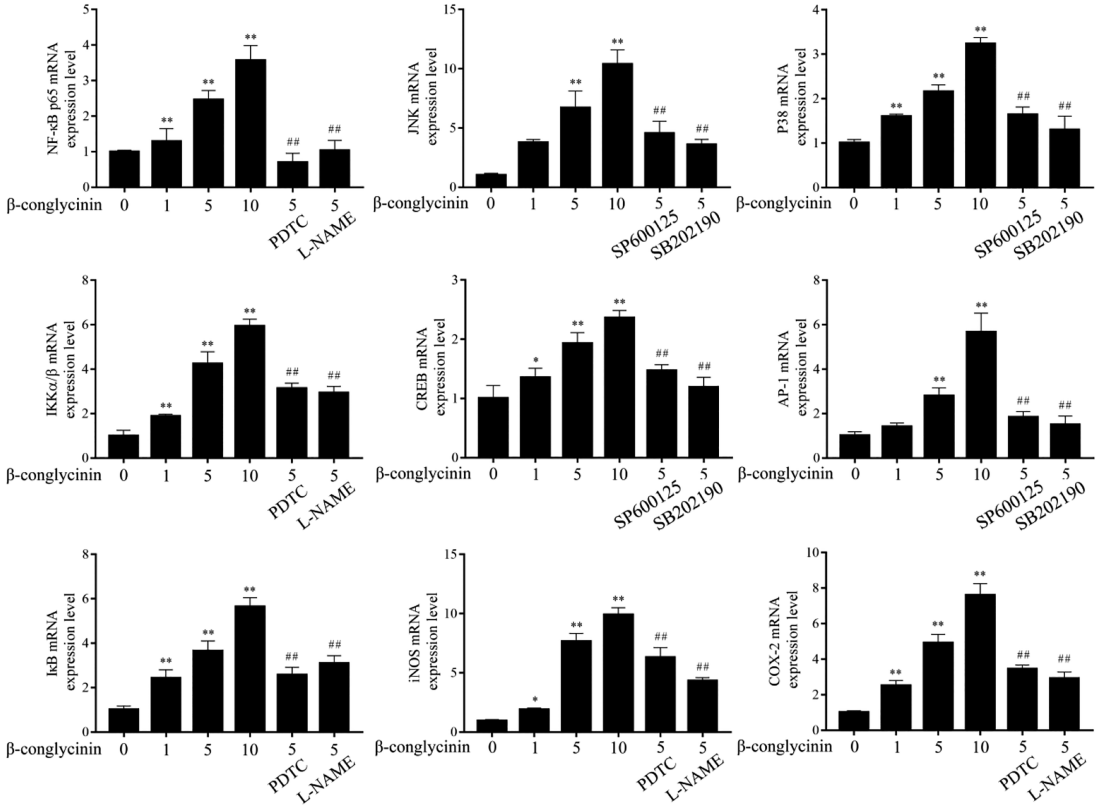

Figure 11. Detection of NF-κB, JNK, p38, and their downstream mRNA expression in IPEC-J2 cells by qRT-PCR after incubation with β-conglycinin or inhibitors. The experiments were repeated in triplicate, and each bar represents the mean ± SD. (*) $p < 0.05$ shows a highly significant difference compared to the control group. (**) $p < 0.01$ shows a highly significant difference compared to the control group. (#) $p < 0.05$ shows a highly significant difference compared to the 5 mg·mL^{-1} β-conglycinin group. (##) $p < 0.01$ shows a highly significant difference compared to the 5 mg·mL^{-1} β-conglycinin group.

and centrifuged (12000g for 10 min). Next, the supernatant was discarded, and 500 μL of 80% ethanol was added to the sample. After centrifugation (7500g for 5 min), the supernatant was discarded. The concentration of RNA was detected with a NanoVue Plus instrument (Thermo Fisher Scientific, Waltham, MA, U.S.A.). Relative gene expression levels were determined by the $2^{-\Delta\Delta C_T}$ method. Primers were designed using Primer Premier 5.0 software and synthesized by Sangon Biotech Co. (Shanghai, China), with reverse transcription of 500 ng of total RNA for each sample (AT341, TransGen, China). The primer sequences used are provided in Table 1.

Western Blotting. IPEC-J2 cells were lysed using 150 μL of radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Biotechnology, Shanghai, China) and afterward centrifuged (12000g for 15 min under 4 °C), and the supernatants were collected. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime Biotechnology, Shanghai, China). All samples were adjusted to 40 μg and then mixed with 5× loading buffer. Sample proteins were separated by the 5% stacking gel and 10−15% separation gel and then transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, U.S.A.), after blocking with 3% bovine serum albumin (BSA) in Tris-buffered saline/Tween-20 buffer under 37 °C for 4 h, followed by incubation with the indicated primary antibodies under 4 °C for 16 h and then further incubation with 1:5000 diluted goat anti-rabbit immunoglobulin G (IgG) secondary antibody (Beijing Biosynthesis Biotechnology, Beijing, China) at 26 °C for 45 min. The results were analyzed using Quantity One one-dimensional analysis software

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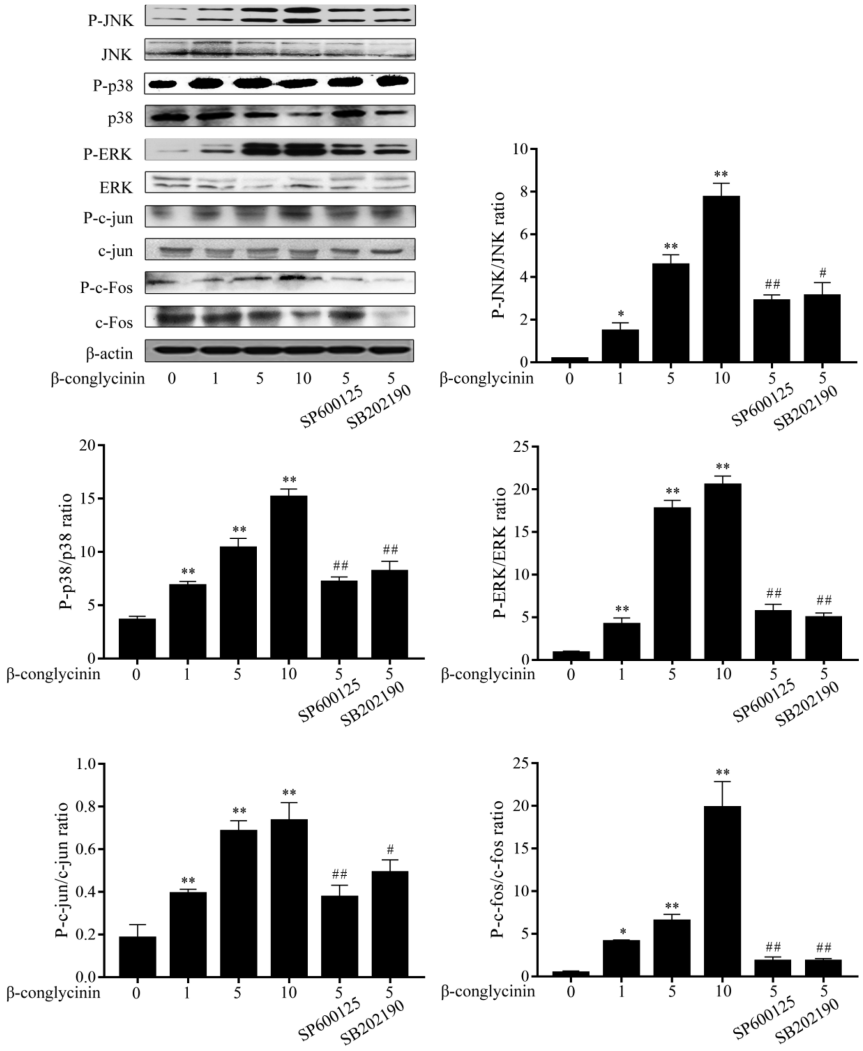

Figure 12. Detection of JNK, p38, ERK, c-Jun, and c-Fos phosphorylation levels in IPEC-J2 cells by western blotting after incubation with β-conglycinin or inhibitors. Each bar represents the mean ± SD of three independent experiments. (*) $p < 0.05$ shows a highly significant difference compared to the control group. (**) $p < 0.01$ shows a highly significant difference compared to the control group. (#) $p < 0.05$ shows a highly significant difference compared to the 5 mg·mL^{-1} β-conglycinin group. (##) $p < 0.01$ shows a highly significant difference compared to the 5 mg·mL^{-1} β-conglycinin group.

(Bio-Rad, Hercules, CA, U.S.A.). The antibodies used are provided in Table 2.

Flow Cytometry. The apoptotic effect of β-conglycinin was quantitatively detected by the annexin V−fluorescein isothiocyanate (FITC) apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, U.S.A.). After cultured with β-conglycinin and inhibitors, the cells were collected and stained with 5 μL of annexin V−FITC and propidium iodide (PI) for 10 min. The signal of cell apoptosis was detected using flow cytometry (BD Biosciences, Franklin Lakes, NJ, U.S.A.), and data were analyzed with FlowJo software (Ashland, OR, U.S.A.).

Cell Immunofluorescence Assay. After treatment with β-conglycinin or inhibitors, cells seeded on sterile glass slides were washed 3 times in phosphate-buffered saline (PBS) for 5 min each time, afterward fixed with 4% polyoxymethylene for 30 min, and then washed 3 times in PBS for 5 min each time. Triton X-100 (0.5%) was used to permeabilize the cell membrane and then blocked with 0.5% BSA for 1 h, and then the primary antibodies against ZO-1, occludin, and claudin-1 were added and incubated with the cells under 4 °C for 16 h. The glass slides were washed 3 times in PBS for 5 min each time, and the FITC-conjugated secondary antibody (dilution of 1:100, E-AB-1016) was added for 2 h. FITC−phalloidin (Abcam, Cambridge, U.K.) was used for staining the cytoskeletal protein (F-actin), which was added for 1 h, then washed 3 times in PBS for 5 min each time. Finally, the cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Abcam, Cambridge, U.K.). The glass slides were then observed with a laser scanning confocal microscope (Olympus, Tokyo, Japan).

Electrophoretic Mobility Shift Assay (EMSA). Cytoplasmic extraction reagents were incubated with a biotin-labeled promoter DNA probe in binding buffer for 30 min on ice. Following incubation, the samples were separated on a 4% polyacrylamide gel in Tris−borate−ethylenediaminetetraacetic acid (EDTA) (NanJing KeyGen Biotech, Nanjing, China) and transferred onto a nylon membrane, and the membrane was fixed by ultraviolet (UV) cross-linking. Finally, the membrane was exposed to X-ray film for 2−5 min. The 25- and 100-fold excess cold probes combined with biotin-labeled probes were used as competition controls and then visualized using a chemiluminescence procedure. The biotin-labeled AP-1, p65, and CREB probes used for the EMSA were provided by NanJing KeyGen Biotech Co., Ltd. (Nanjing, China). The sequences of the consensus oligonucleotide were as follows: NF-κB p65 forward, 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ and NF-κB p65 reverse, 3′-TCA ACT CCC CTG AAA GGG TCC G-5′; CREB forward, 5′-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3′ and CREB reverse, 3′-TCT CTA ACG GAC TGC AGT CTC TCG ATC-5′; and AP-1 forward, 5′-CGC TTG ATG ACT CAG CCG GAA-3′ and AP-1

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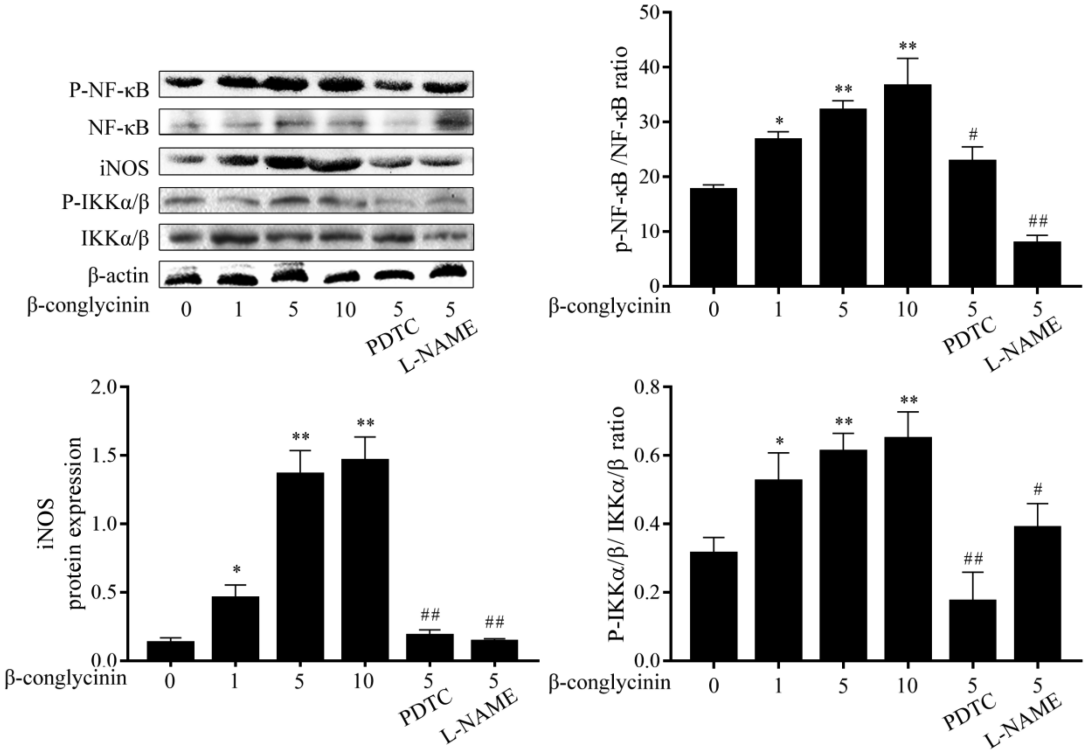

Figure 13. Detection of NF-κB and IKKα/β phosphorylation and iNOS protein expression levels in IPEC-J2 cells by western blotting after incubation with β-conglycinin or inhibitors. Each bar represents the mean ± SD of three independent experiments. (*) $p < 0.05$ shows a highly significant difference compared to the control group. (**) $p < 0.01$ shows a highly significant difference compared to the control group. (#) $p < 0.05$ shows a highly significant difference compared to the 5 mg·mL^{-1} β-conglycinin group. (##) $p < 0.01$ shows a highly significant difference compared to the 5 mg·mL^{-1} β-conglycinin group.

reverse, 3′-GCG AAC TAC TGA GTC GGC CTT-5′. Protein−DNA binding reactions were performed using a chemiluminescent EMSA kit (KGS101, NanJing KeyGen Biotech Co., Ltd.) according to the protocol of the manufacturer.

Statistical Analysis. Each experiment was repeated in triplicate. Analysis of variance was used to evaluate differences within each group, using IBM SPSS Statistics 20 (SPSS, Inc., Chicago, IL, U.S.A.). Data were reported as the mean ± standard deviation (SD), with a two-sided 5% significance level.

RESULTS

Purity of β-Conglycinin. Figure 1 shown that, after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE) analysis, β-conglycinin contained three bands, including α′, α, and β subunits. The purity of β-conglycinin was 90%.

Viability of IPEC-J2 Cells after β-Conglycinin Exposure. As shown in Figure 2, cell viability was significantly decreased after adding β-conglycinin. However, cell viability was increased dramatically in the 5 mg·mL^{-1} β-conglycinin + 1 μmol·L^{-1} NF-κB inhibitor (PDTC), iNOS inhibitor (L-NAME), JNK inhibitor (SP600125), and p38 inhibitor (SB202190) groups compared to the 5 mg·mL^{-1} β-conglycinin group.

ELISA Results. As shown in Figure 3, the levels of cytokine, caspase-3/-8, and COX-2 were significantly enhanced after adding β-conglycinin to IPEC-J2 cells ($p < 0.01$). However, their levels were significantly decreased in the 5 mg·mL^{-1} β-conglycinin + 1 μmol·L^{-1} NF-κB inhibitor (PDTC), iNOS inhibitor (L-NAME), JNK inhibitor (SP600125), and p38 inhibitor (SB202190) groups compared to the 5 mg·mL^{-1} β-conglycinin group.

Alkaline Phosphatase Activity. As shown in Figure 4, alkaline phosphatase activity significantly increased proportionally to β-conglycinin levels ($p < 0.01$). In comparison to the 5 mg·mL^{-1} β-conglycinin group, alkaline phosphatase activity decreased significantly after 1 μmol·L^{-1} NF-κB inhibitor (PDTC), iNOS inhibitor (L-NAME), JNK inhibitor (SP600125), or p38 inhibitor (SB202190) addition ($p < 0.01$).

Transmission Electron Microscopic Images of IPEC-J2 Cells. Electron microscopy images are shown in Figure 5. In the control group, IPEC-J2 cells displayed complete nuclei with homogeneously dispersed chromatin. In the 5 mg·mL^{-1} β-conglycinin group, the IPEC-J2 cells exhibited mitochondrial alterations, which were considered to be a typical ultrastructural feature of apoptosis. In the 10 mg·mL^{-1} β-conglycinin group, small vesicles were observed near the nucleus, and the nucleus was broken up into several discrete fragments. The entire cellular morphology of granulosa cells was disrupted; highly condensed chromatin was present marginally near the inner nuclear membrane; the nuclear lamina had degenerated along with most of the cytoplasmic organelles; the nucleolus was enlarged; and its granules were coarse and scattered. Cytoplasm had begun to condense, while the organelles remained intact.

In comparison to the 5 mg·mL^{-1} β-conglycinin group, the structure of IPEC-J2 cells in the 5 mg·mL^{-1} β-conglycinin + 1 μmol·L^{-1} NF-κB inhibitor (PDTC), iNOS inhibitor (L-NAME), JNK inhibitor (SP600125), and p38 inhibitor (SB202190) groups tended to be complete and regular, the cytoplasm was denser, organelles were more intact, and lysosomes were scattered. The chromatin distribution in the nucleus was more uniform.

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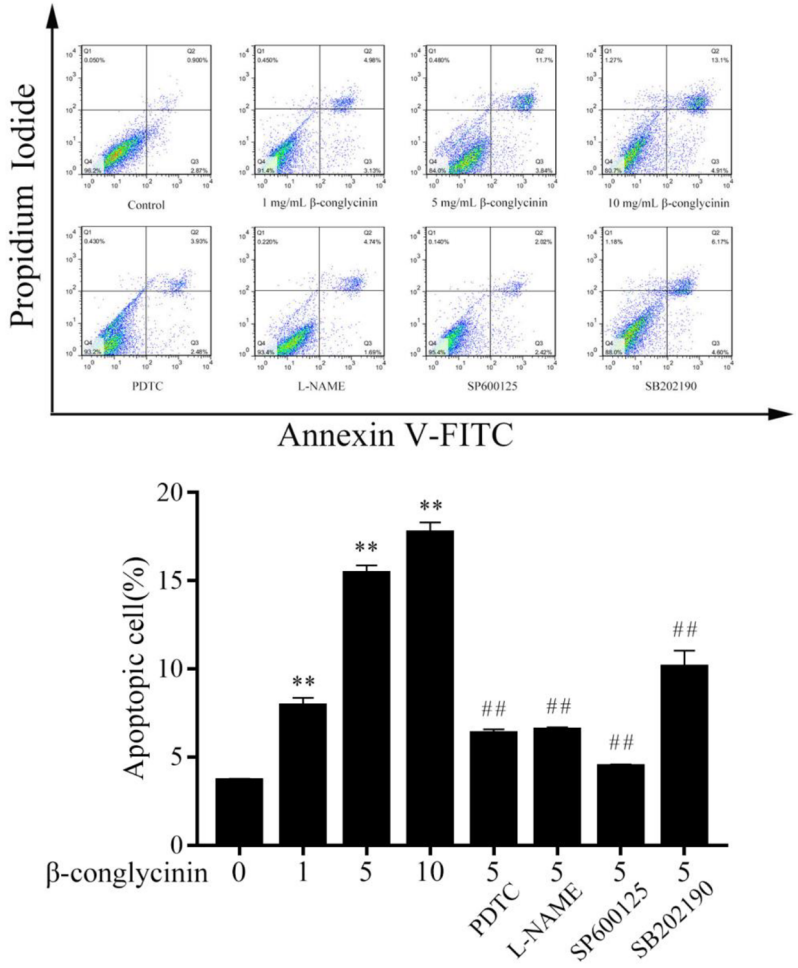

Figure 14. Detection of the IPEC-J2 cell apoptosis rate by flow cytometry analysis after incubation with β-conglycinin or inhibitors. The experiments were repeated in triplicate, and each bar represents the mean ± SD. (*) $p < 0.05$ shows a highly significant difference compared to the control group. (**) $p < 0.01$ shows a highly significant difference compared to the control group. (#) $p < 0.05$ shows a highly significant difference compared to the 5 mg·mL^{-1} β-conglycinin group. (##) $p < 0.01$ shows a highly significant difference compared to the 5 mg·mL^{-1} β-conglycinin group.

Effect of β-Conglycinin on TJ Protein in IPEC-J2 Cells. The TJ proteins (occludin, claudin-1, and ZO-1) was assessed by western blotting, qRT-PCR, and immunofluorescence. Cytoskeleton protein expression was detected by immunofluorescence. The effects of β-conglycinin on TJ proteins in IPEC-J2 cells are shown in Figure 6. β-Conglycinin-treated groups showed a significant decrease in protein and mRNA expression of claudin-1, ZO-1, and occludin ($p < 0.01$ or $p < 0.05$). In comparison to the 5 mg·mL^{-1} β-conglycinin group, the protein and mRNA expression of claudin-1, ZO-1, and occludin in IPEC-J2 cells in the 5 mg·mL^{-1} β-conglycinin + 1 μmol·L^{-1} of NF-κB inhibitor (PDTC), iNOS inhibitor (L-NAME), JNK inhibitor (SP600125), or p38 inhibitor (SB202190) groups were significantly increased ($p < 0.01$ or $p < 0.05$).

As shown in Figures 7−9, after culturing the cells with β-conglycinin, the immunofluorescence signal of the TJ proteins in IPEC-J2 cells significantly weakened and the cell connection location was decreased. The protein expression of occludin, claudin-1, and ZO-1 was significantly decreased in comparison to the control group ($p < 0.05$). The TJ proteins in the 5 mg·mL^{-1} β-conglycinin + 1 μmol·L^{-1} NF-κB inhibitor (PDTC), iNOS inhibitor (L-NAME), JNK inhibitor (SP600125), or p38 inhibitor (SB202190) groups tended to be complete and more regular than in the 5 mg·mL^{-1} β-conglycinin group. The TJ proteins were located at cell−cell junction regions.

Effect of β-Conglycinin on the Cytoskeleton in IPEC-J2 Cells. As shown in Figure 10, the cytoskeleton of IPEC-J2 cells in the 1 mg·mL^{-1} β-conglycinin group was slightly damaged, while those in the 5 and 10 mg·mL^{-1} β-conglycinin groups were severely damaged. However, the addition of 5 mg·mL^{-1} β-conglycinin with inhibitors (PDTC, L-NAME, SP600125, and SB202190) resulted in only slight damage to the integrity of the cytoskeleton.

Effect of β-Conglycinin on the mRNA Expression of NF-κB, iNOS, JNK, and p38 and Their Downstream Genes. Figure 11 showed a significant linear increase in the mRNA expression of NF-κB, iNOS, JNK, and p38, and their downstream genes were detected after incubation with 0, 1, 5, and 10 mg·mL^{-1} β-conglycinin at 24 h ($p < 0.01$ or $p < 0.05$). Treatment with the inhibitors (PDTC and L-NAME) significantly inhibited the upregulated expression of NF-κB, iNOS, JNK, and p38, and their downstream genes were induced by β-conglycinin ($p < 0.01$ or $p < 0.05$).

Effect of β-Conglycinin on NF-κB/MAPK-Related Protein Expression. Western blotting results are shown in Figures 12 and 13. β-Conglycinin treatment increased the

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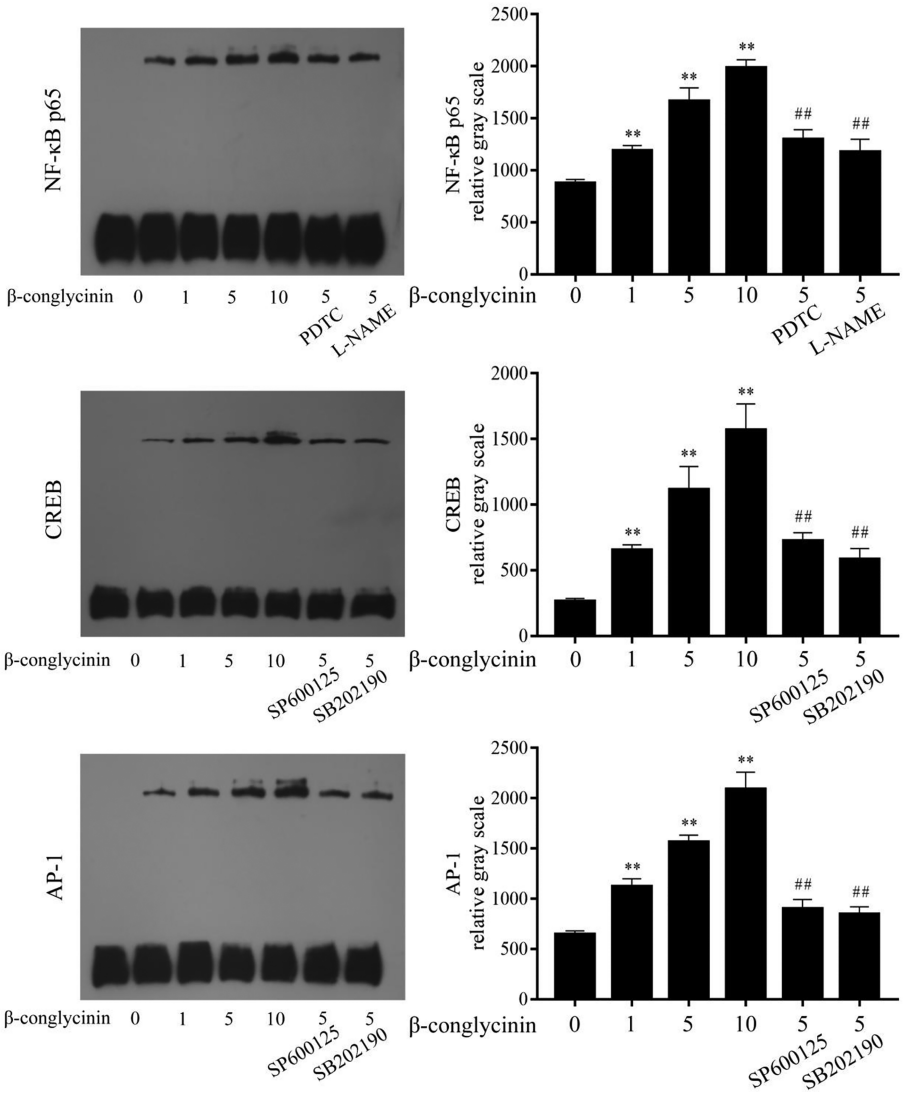

Figure 15. Detection of NF-κB p65, CREB, and AP-1 DNA-binding activity by EMSA analysis after incubation with β-conglycinin or inhibitors. The experiments were repeated in triplicate, and each bar represents the mean ± SD. (*) $p < 0.05$ shows a highly significant difference compared to the control group. (**) $p < 0.01$ shows a highly significant difference compared to the control group. (#) $p < 0.05$ shows a highly significant difference compared to the 5 mg·mL^{-1} β-conglycinin group. (##) $p < 0.01$ shows a highly significant difference compared to the 5 mg·mL^{-1} β-conglycinin group.

phosphorylation of JNK, p38, ERK, NF-κB, c-Fos, c-Jun, and IKKα/β ($p < 0.01$ or $p < 0.05$). However, these levels were decreased after adding 1 μmol·L^{-1} NF-κB inhibitor (PDTC), iNOS inhibitor (L-NAME), JNK inhibitor (SP600125), or p38 inhibitor (SB202190) ($p < 0.01$ or $p < 0.05$).

IPEC-J2 Cell Apoptosis Rate by Flow Cytometry Analysis after 24 h of Incubation with β-Conglycinin. Figure 14 showed that β-conglycinin triggered IPEC-J2 cell apoptosis. The apoptosis rate significantly increased with increasing β-conglycinin levels ($p < 0.01$) and decreased after adding 1 μmol·L^{-1} NF-κB inhibitor (PDTC), iNOS inhibitor (L-NAME), JNK inhibitor (SP600125), or p38 inhibitor (SB202190) ($p < 0.01$).

Effect of β-Conglycinin on NF-κB p65, CREB, and AP-1 DNA-Binding Activities. As shown in Figure 15, the EMSA results showed that the NF-κB p65, CREB, and AP-1 DNA-binding activities were enhanced in a dose-dependent manner with increasing concentrations of β-conglycinin ($p < 0.01$) and decreased after adding 1 μmol·L^{-1} NF-κB inhibitor (PDTC), iNOS inhibitor (L-NAME), JNK inhibitor (SP600125), or p38 inhibitor (SB202190) ($p < 0.01$).

DISCUSSION

The intestinal epithelium forms an essential interface with the luminal environment and against harmful components, which controls the ingestion of nutrients.[29,30] The present study investigated β-conglycinin-induced damage to the intestinal epithelium using IPEC-J2 cells. Our TEM, immunofluorescence, and flow cytometry analyses suggested that increasing levels of β-conglycinin caused the TJ and cytoskeleton to loosen, augmented epithelial permeability, and then induced IPEC-J2 cell damage and apoptosis, similar to the result shown by Chen et al.[31] Alkaline phosphatase activity reflects cellular membrane integrity. The concentration of alkaline phosphatase in the supernatant increased, indicating that β-conglycinin damaged the cell membrane. Additionally, flow cytometry analysis showed that β-conglycinin significantly increased the ratio of apoptotic cells, whereas NF-κB, iNOS, JNK, and p38 inhibitors efficiently

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inhibited apoptosis induced by β-conglycinin. Zhao et al. determined the epithelial permeability, integrity, and TJ expression and distribution in IPEC-J2 cells incubated with β-conglycinin, separately;[15] their results demonstrated that the altered TJ expression caused by β-conglycinin results in barrier dysfunction of the intestinal epithelium, which was similar to our findings, but the mechanism remains unclear. Our previous study indicated that β-conglycinin damaged intestinal function in piglets by altering NF-κB, JNK, and p38 expression,[32] and further *in vitro* studies are needed to confirm the generality of our findings.

In the present study, inhibitors of NF-κB (PDTC), iNOS (L-NAME), JNK (SP600125), and p38 (SB202190) were used to investigate the underlying mechanisms of action of β-conglycinin-induced IPEC-J2 cell damage. The phosphorylation levels of NF-κB, JNK, p38, ERK, and their downstream proteins were significantly increased; however, the phosphorylation levels were significantly reduced after adding the inhibitors. The relative mRNA expression levels of apoptosis-related genes, including IKKβ, iNOS, and IKKα, were consistent with the protein measurements. Moreover, IPEC-J2 cellular activity was improved when β-conglycinin-induced apoptosis was prevented by the inhibitors. These results suggested that β-conglycinin induced IPEC-J2 cell damage through the MAPK/NF-κB signaling pathway.

The NF-κB/MAPK signaling pathway is responsible for controlling the expression of mediators and pro-inflammatory cytokines.[33,34] Modulation of cell functions by MAPKs is mediated primarily by phosphorylation of their downstream substrates, including several transcription factors (such as AP-1). Additionally, AP-1 is mediated by ERK1/2, JNK, and other signaling molecules, which control many cellular processes, including differentiation, proliferation, and apoptosis.[35] Our EMSA results indicated that β-conglycinin enhanced the DNA-binding activity of AP-1, and qRT-PCR revealed that β-conglycinin upregulated the relative mRNA expression levels of AP-1; however, after adding JNK or p38 inhibitors, the DNA-binding activity and corresponding mRNA of AP-1 expression levels decreased. Increased AP activity indicated that cellular integrity was destroyed by β-conglycinin, which corresponds to the changes in the TJ proteins and cytoskeleton. CREB is a transcription factor that targets multiple signaling pathways and mediates cellular responses to extracellular stimuli, including proliferation, differentiation, and adaptive cellular responses.[36] In this study, the DNA-binding activity and relative mRNA expression levels of CREB remarkably increased after treatment with β-conglycinin. However, the DNA-binding activity and relative mRNA expression levels of CREB and AP-1 significantly decreased when JNK (SP600125) or p38 (SB202190) inhibitors were added to IPEC-J2 cells.

Moreover, MAPK activation is essential for regulating NF-κB activity.[20] In our study, the phosphorylation levels and DNA-binding activity of NF-κB were significantly increased after treatment with β-conglycinin. However, the phosphorylation levels and DNA-binding activity of NF-κB were significantly decreased when NF-κB (PDTC) and iNOS (L-NAME) inhibitors were added to IPEC-J2 cells, which is consistent with the results observed for CREB and AP-1. These results indicated that the expression of TJ proteins and cytoskeleton proteins was increased and the expression of apoptosis-related genes and the ratio of apoptotic cells were decreased when the NF-κB/MAPK signaling pathway were inactive. However, the effects of processing and digestion on permeability should be explored in future research, because they may influence allergic reactions.

In this study, IPEC-J2 cells were employed as a intestinal epithelium model to study the effects of β-conglycinin on the cells and to identify β-conglycinin allergen-related signaling pathways. In summary, the results of this study indicated that β-conglycinin caused damage to IPEC-J2 cells via the NF-κB/MAPK signaling pathway. This finding is crucial for exploring the mechanism of allergic reactions caused by soybean antigen proteins.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

AP, activator protein; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; iNOS, inducible nitric oxide synthase; IPEC-J2 cells, intestinal porcine epithelial cells; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor κB; PDTC, pyrrolidine dithiocarbamate; PVDF, polyvinylidene fluoride; TEM, transmission electron microscopy; TJ, tight junction

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