Regulation of Immune Stress in Piglet Intestinal Epithelial Cells by Achyranthes bidentata Polysaccharide and Its Mechanism of Action: Postprint

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Abstract

This experiment aimed to investigate the effects of Achyranthes bidentata polysaccharide (ABPS) on the secretion and expression of pro-inflammatory cytokines in porcine jejunal epithelial cells (IPEC-J2) under lipopolysaccharide (LPS)-induced immune stress, and to explore the potential mechanisms by which ABPS regulates immune stress in IPEC-J2. IPEC-J2 cells at passages 4-5 were cultured in medium supplemented with 0 (control), 600, 900, or 1200 g/mL ABPS and 10 g/mL LPS, with 12 replicates per group (one well per replicate). Following 72 h of culture, enzyme-linked immunosorbent assay (ELISA) was employed to detect the effects of ABPS on the secretion of pro-inflammatory cytokines including interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), and tumor necrosis factor-α (TNF-α). Quantitative real-time PCR was used to determine the mRNA expression levels of Toll-like receptor 4 (TLR4) and nuclear transcription factor- B (NF- B), while Western blotting was utilized to measure the protein expression levels of TLR4, NF- B, and phosphorylated nuclear transcription factor- B (p-NF- B). The results demonstrated that, compared with the control group, the 300, 600, 900, and 1200 g/mL ABPS groups significantly reduced the secretion of IL-1, IL-6, IL-8, and TNF-α ($P<0.05$). The 300 g/mL ABPS group significantly decreased p-NF- B protein expression ($P<0.05$), whereas the 900 and 1200 g/mL ABPS groups significantly reduced TLR4 and NF- B mRNA expression as well as NF- B protein expression ($P<0.05$). These findings indicate that ABPS regulates pro-inflammatory cytokine secretion via the TLR4/NF- B signaling pathway, thereby alleviating immune stress. Low-concentration ABPS reduces immune stress by directly inhibiting NF- B phosphorylation, whereas high-concentration ABPS alleviates immune stress by suppressing the expression of TLR4 mRNA, NF- B mRNA, and NF- B protein.

Full Text

Achyranthes bidentata Polysaccharides: Regulation of Immunological Stress in Piglet Intestinal Epithelial Cells and Its Mechanism of Action

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Abstract: This study investigated the effects of *Achyranthes bidentata* polysaccharides (ABPS) on the secretion and expression of pro-inflammatory cytokines in piglet jejunum epithelial cells (IPEC-J2) under lipopolysaccharide (LPS)-induced immunological stress and explored the potential mechanisms underlying ABPS regulation of IPEC-J2 immune stress. IPEC-J2 cells at passages 4–5 were cultured with 0 (control), 600, 900, or 1,200 μg/mL ABPS and 10 μg/mL LPS, with 12 replicates per group (one well per replicate). After 72 h of culture, enzyme-linked immunosorbent assay (ELISA) was used to detect secretions of pro-inflammatory cytokines interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 8 (IL-8), and tumor necrosis factor α (TNF-α). Real-time quantitative PCR measured mRNA expression of Toll-like receptor 4 (TLR4) and nuclear factor B (NF- B), while Western blot analysis determined protein expression levels of TLR4, NF- B, and phosphorylated NF- B (p-NF- B). Compared with the control group, ABPS at 300, 600, 900, and 1,200 μg/mL significantly reduced secretions of IL-1, IL-6, IL-8, and TNF-α ($P < 0.05$). The 300 μg/mL ABPS group significantly decreased p-NF- B protein expression ($P < 0.05$), while 900 and 1,200 μg/mL ABPS groups significantly reduced mRNA expression of TLR4 and NF- B as well as NF- B protein expression ($P < 0.05$). These results demonstrate that ABPS regulates pro-inflammatory cytokine secretion and alleviates immune stress via the TLR4/NF- B signaling pathway. Low-concentration ABPS reduces immune stress by directly inhibiting NF- B phosphorylation, whereas high-concentration ABPS alleviates immune stress by suppressing TLR4 mRNA, NF- B mRNA, and NF- B protein expression.

Keywords: *Achyranthes bidentata* polysaccharides; lipopolysaccharide; pro-inflammatory cytokines; Toll-like receptor 4; nuclear factor B

Introduction

The intestine serves as the primary organ for digestion and nutrient absorption and constitutes a vital component of the immune system. Intestinal function directly impacts animal health [1]. Intestinal epithelial cells play crucial physiological roles in the intestinal lumen, not only absorbing nutrients but also sensing

foreign stimuli and participating in intestinal immune responses [2]. Research indicates that lipopolysaccharide (LPS), a metabolic product of Gram-negative bacteria, is a major factor causing intestinal dysfunction in pigs. Its mechanism of action involves activating the Toll-like receptor 4 (TLR4)/nuclear factor B (NF- B) signaling pathway in intestinal epithelial cells, triggering intracellular inflammatory responses and promoting massive release of pro-inflammatory cytokines [3-4]. Recent studies suggest that plant extracts represent potential nutritional additives that can improve intestinal function. *Achyranthes bidentata* polysaccharides (ABPS) are water-soluble small molecular neutral polysaccharides extracted, isolated, and purified from the roots of *Achyranthes bidentata* (Amaranthaceae), possessing immune-enhancing and anti-inflammatory functions [5-6]. Animal trials have demonstrated that ABPS supplementation improves intestinal function in piglets and reduces circulating pro-inflammatory cytokine concentrations [7-8]. Yuan et al. [9] reported that astragalus polysaccharides protect intestinal health by inhibiting NF- B activation through the TLR4/NF- B signaling pathway, thereby reducing pro-inflammatory cytokine release. However, the molecular mechanisms through which ABPS improves intestinal function remain unclear. As ABPS is also a polysaccharide, we hypothesized that ABPS influences pro-inflammatory cytokine release and improves intestinal health via the TLR4/NF- B signaling pathway.

This study employed piglet jejunum epithelial cells (IPEC-J2) as a model to investigate the effects of ABPS on inflammatory cytokines in LPS-stressed IPEC-J2 cells and to determine whether IPEC-J2 alleviates immune stress through the TLR4/NF- B signaling pathway. The findings will enhance understanding of the molecular mechanisms underlying ABPS-mediated intestinal protection and provide a theoretical basis for the application of ABPS in animal production.

Materials and Methods

1.1 Cell Line IPEC-J2 cells were generously provided by the Institute of Subtropical Agriculture, Chinese Academy of Sciences.

1.2 Reagents and Materials ABPS (purity 98%) was purchased from Xi'an Tianrui Technology Biological Co., Ltd. LPS was obtained from Sigma-Aldrich. DMEM/F12 culture medium, 0.25% porcine trypsin, and antibiotic-antimycotic solution were purchased from Hyclone. Fetal bovine serum (FBS) and cell cryopreservation medium were from Gibco. Phosphate-buffered saline (PBS) was from Biotopped.

1.3 Cell Culture IPEC-J2 cells at passages 4–5 were cultured in DMEM/F12 medium supplemented with 10% FBS and 1% antibiotics in a humidified incubator at 37 °C with 5% CO_2. Logarithmic-phase IPEC-J2 cells were treated with 0 (control), 600, 900, or 1,200 μg/mL ABPS and 10 μg/mL LPS. Each group contained 12 replicates (one 12-well plate), with each well serving as one replicate and seeded at identical cell density. After 72 h of culture, cells were washed

three times with pre-warmed PBS (37 °C) and digested with pre-warmed 0.25% trypsin for 3 min, followed by reaction termination with DMEM/F12 medium.

1.4 Measurement of IPEC-J2 Cytokine Secretions Enzyme-linked immunosorbent assay (ELISA) was used to determine secretions of interleukin 1 (IL-1), interleukin 6 (IL-6), interleukin 8 (IL-8), and tumor necrosis factor α (TNF-α) according to kit instructions. After cell lysis, prepared reagents were equilibrated at room temperature (18–25 °C) for at least 30 min. Standards and samples were added to coated microplates and incubated at 37 °C for 2 h. Following liquid removal and drying, biotin-labeled working solution was added and incubated at 37 °C for 1 h. After washing three times with PBS and drying, horseradish peroxidase (HRP)-labeled avidin was added and incubated at 37 °C for 1 h. After five PBS washes and drying, substrate solution was added and color was developed at 37 °C for 15–30 min in the dark. Stop solution was added to terminate the reaction, and optical density (OD) values were measured at 450 nm using a microplate reader 5 min after termination.

1.5 Real-Time Quantitative PCR (RT-qPCR) Analysis of TLR4 and NF- B mRNA Expression Following trypsin digestion and centrifugation, total RNA was extracted using Trizol reagent (Invitrogen, USA) and dissolved in ultrapure water. RNA integrity was verified by 1% agarose gel electrophoresis [Figure 1: see original paper], confirming absence of DNA contamination. RNA purity was assessed by spectrophotometry at 260 and 280 nm; samples with OD_{260}/OD_{280} ratios of 1.8–2.0 were accepted for analysis.

[Figure 1: see original paper]

Equal concentrations of total RNA were used for reverse transcription according to the kit instructions (Beijing Kangwei Century) in a 30 µL reaction system. Complementary DNA (cDNA) products were stored at -20 °C for real-time quantitative PCR. Target gene sequences were retrieved from NCBI, and primers were designed using Primer5 software. Primer sequences and parameters are listed in Table 1 . SYBR Green I chemistry was employed on a PIKO REAL 96 real-time PCR system (Thermo). Each sample was run in triplicate (three wells per indicator) in a 30 µL system containing: 1 µL cDNA, 0.5 µL each of forward and reverse primers (10 µmol/L), 15 µL 2× SYBR Green PCR Master Mix, and nuclease-free water to 30 µL. Amplification conditions were: 50 °C for 3 min; 95 °C for 10 min; 40 cycles of 95 °C for 5 s and 60 °C for 30 s. A melting curve was generated from 60 to 95 °C after amplification. Relative mRNA expression was calculated using the $2^{-}\Delta\Delta$Ct method normalized to the reference gene β-actin using the following formulas: ΔCt = Ct(target gene) -Ct(reference gene);
-$\Delta\Delta$Ct = ΔCt(control group) -ΔCt(treatment group);
mRNA expression level = $2^{-}\Delta\Delta$Ct.

Table 1 Parameters of primers for RT-qPCR

Genes	Primer sequences (5' – 3')	Amplification length/bp	GenBank accession No.
β-actin	Forward: CATCCTGCGTCTG-GACCTGGReverse: TAATGTCACGCAC-GATTTCC		NM_{001101}.3
TLR4	Forward: CCACCTGTCA-GATAAGCGReverse: CCTCAC-CCAGTCTTCGTC		NM_{001113039}.2
NF- B	Forward: AAGAGCAGCGTG-GTGGGCAGTGRe-verse: CCGGAACGGTCTC-CATCACAATC		NM_{001048232}.1

1.6 Western Blot Analysis of TLR4, NF- B, and p-NF- B Protein Expression After washing, cells were centrifuged for 2 min and the supernatant was discarded. Lysis buffer was added and mixed, followed by protein extraction on ice for 30 min. Samples were centrifuged at 12,000 rpm for 15 min at 4 °C. Bicinchoninic acid (BCA) solution was prepared at a 50:1 ratio. Protein standard (2 mg/mL) was added to a 96-well plate at volumes of 0, 1, 2, 3, 4, 5, and 6 µL, adjusted to 20 µL with distilled water. Samples were added to the plate and adjusted to 20 µL, followed by addition of 200 µL BCA solution to each well and incubation at 37 °C for 30 min. Protein concentrations were determined from a standard curve. Fifty to 100 µg of protein was mixed with 5× loading buffer, denatured by boiling for 5 min, and snap-frozen. Denatured proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 12% separation gel and 5% stacking gel at 80 V (stacking) and 120 V (separation). Electrophoresis was terminated when bromophenol blue reached the gel bottom. Proteins were transferred to nitrocellulose membranes via semi-dry transfer, blocked, and washed. Primary antibodies were diluted in 1× TBST and incubated with membranes overnight at 4 °C. After three 15-min washes with 1× TBST, membranes were incubated with HRP-conjugated secondary antibodies (Proteintech) diluted 1:3,000 in 1× TBST for 45–60 min. Following three additional 15-min washes, membranes were incubated with ECL chemiluminescent reagent (Thermo) for 3 min, exposed to X-ray film for several seconds to minutes, and developed. Protein expression levels were calculated as the ratio of target protein band intensity to internal reference protein band intensity.

1.7 Statistical Analysis Data were analyzed using SPSS 17.0 statistical software. Multiple comparisons of means were performed using Duncan' s method.

Results

2.1 Effects of ABPS on Cytokine Secretions in LPS-Stressed IPEC-J2 Cells Table 2 shows that compared with the control group, ABPS at 300, 600, 900, and 1,200 μg/mL significantly reduced secretions of IL-1, IL-6, IL-8, and TNF-α ($P < 0.05$). Significant differences in cytokine secretions were observed among the 300, 600, 900, and 1,200 μg/mL ABPS groups ($P < 0.05$), with IL-1, IL-6, IL-8, and TNF-α secretions showing a dose-dependent decrease as ABPS concentration increased.

Table 2 Effect of ABPS on cytokine secretions in LPS-stressed IPEC-J2 cells

Items	ABPS concentration/(μg/mL)					P-value
	0	300	600	900	1,200	
IL-1	48.25$±0.46^a\|45.06±0.32^b\|42.73±[illegible]^c\|603.05±10.77^d|529.45±					0.0001||$IL-$
	6|15.43±0.21^a|13.30±0.14^b|8.76±0.15^c|7.96±0.20^d|7.44±0.15^e| <					0.0001||$IL-$
	8|764.93±9.35^a|670.79±6.13^b|609.67±9.09^c|531.59±11.04^d|478.42±9.13^e| <					0.0001||$TNF-$ \$

Values in the same row with different letter superscripts differ significantly ($P < 0.05$).

2.2 Effects of ABPS on TLR4 and NF- B mRNA Expression in LPS-Stressed IPEC-J2 Cells Figure 2 [Figure 2: see original paper] demonstrates that compared with the control group, 900 and 1,200 μg/mL ABPS significantly decreased mRNA expression of TLR4 and NF- B ($P < 0.05$), whereas 300 μg/mL ABPS significantly increased their expression ($P < 0.05$). The 600 μg/mL ABPS group showed no significant difference from the control ($P > 0.05$) but exhibited lower expression levels. TLR4 and NF- B mRNA expression displayed a dose-dependent relationship with ABPS concentration, decreasing as ABPS concentration increased.

[Figure 2: see original paper]

2.3 Effects of ABPS on TLR4, NF- B, and p-NF- B Protein Expression in LPS-Stressed IPEC-J2 Cells Figure 3 [Figure 3: see original paper] shows that compared with the control group, 300 μg/mL ABPS significantly increased TLR4 protein expression ($P < 0.05$), while 600, 900, and 1,200 μg/mL ABPS showed no significant differences ($P > 0.05$). The 1,200 μg/mL ABPS group significantly decreased NF- B protein expression ($P < 0.05$), whereas 300,

600, and 900 μg/mL ABPS groups significantly increased NF- B protein expression ($P < 0.05$). The 300 μg/mL ABPS group significantly reduced p-NF- B protein expression ($P < 0.05$), while 600, 900, and 1,200 μg/mL ABPS groups showed no significant differences from the control ($P > 0.05$).

[Figure 3: see original paper]

Discussion

Intestinal epithelial cells constitute the first line of defense against pathogen invasion through the gut. The IPEC-J2 cell line, derived from the jejunum of 1-day-old piglets, is commonly used as an intestinal epithelial cell model to study microbial infections [10-11]. Numerous studies have shown that intestinal epithelial cells secrete pro-inflammatory cytokines such as IL-1, IL-6, IL-8, and TNF-α when invaded by pathogenic microorganisms or stimulated by LPS [12-14]. Our results confirm that LPS induces pro-inflammatory cytokine secretion in IPEC-J2 cells. Excessive release of pro-inflammatory cytokines can induce apoptosis in intestinal epithelial cells, enhance tissue damage mediated by cellular and humoral immunity, recruit neutrophils and other inflammatory cells to intestinal lesions, and trigger intestinal inflammatory responses and tissue destruction, ultimately leading to diarrhea and edema in piglets [4]. Therefore, reducing excessive pro-inflammatory cytokine secretion benefits piglet intestinal health. ABPS exerts immunomodulatory and anti-inflammatory effects by regulating pro-inflammatory cytokine release. Chen et al. [15] and CHEN et al. [16] reported that ABPS increased serum TNF-α, IL-1β, and IL-6 secretions and upregulated IL-1β gene expression in liver, jejunal mucosa, and mesenteric lymph nodes of piglets. Wang et al. [17] found that dietary ABPS supplementation increased serum TNF-α in grass carp. However, when animals undergo LPS-induced immune stress resulting in massive pro-inflammatory cytokine release, ABPS can inhibit cytokine secretion. Zhu et al. [18] demonstrated that ABPS significantly alleviated LPS-induced TNF-α elevation in piglet plasma. Zhang [7] similarly reported that ABPS suppressed LPS-induced increases in pro-inflammatory cytokine secretions in piglet plasma. Xu [19] found that ABPS reduced TNF-α activity and decreased inflammatory responses in mice. While animal trials have shown that ABPS improves piglet intestinal function, whether ABPS exerts immunomodulatory and anti-inflammatory effects by regulating pro-inflammatory cytokine release in intestinal epithelial cells remains unclear. Our findings demonstrate that appropriate ABPS concentrations inhibit LPS-induced IL-1, IL-6, IL-8, and TNF-α secretion in IPEC-J2 cells, providing theoretical evidence for ABPS-mediated alleviation of intestinal immune stress and establishing a foundation for further investigation into its immunoregulatory mechanisms.

TLR4 is a transmembrane receptor in the innate immune system and the primary recognition receptor for LPS [20]. Upon LPS stimulation, TLR4 transmits signals intracellularly to activate NF- B through both myeloid differentiation factor 88 (MyD88)-dependent and -independent pathways [21], leading to mas-

sive pro-inflammatory factor release and initiation of inflammatory responses. NF- B is a pleiotropic nuclear transcription factor downstream of TLR4 that regulates cell proliferation, apoptosis, and immune-inflammatory reactions [22]. Important pro-inflammatory cytokines involved in inflammatory responses, including TNF-α, IL-1, and IL-6, are confirmed to be regulated by NF- B [23]. Studies have shown that certain plant polysaccharides exert anti-inflammatory and immunomodulatory effects by inhibiting NF- B activation [24-26]. Our results demonstrate that high-concentration ABPS significantly reduced mRNA expression of TLR4 and NF- B and NF- B protein expression under LPS stress, while low-concentration ABPS significantly decreased p-NF- B protein expression. These findings indicate that ABPS inhibits LPS-induced pro-inflammatory cytokine release in IPEC-J2 cells and alleviates immune stress-induced intestinal damage through the TLR4/NF- B signaling pathway. However, different ABPS concentrations act through distinct mechanisms: low-concentration ABPS reduces immune stress by directly inhibiting NF- B phosphorylation, whereas high-concentration ABPS alleviates immune stress by suppressing TLR4 mRNA, NF- B mRNA, and NF- B protein expression.

Conclusion

ABPS regulates pro-inflammatory cytokine secretion and alleviates immune stress through the TLR4/NF- B signaling pathway. Low-concentration ABPS reduces immune stress by directly inhibiting NF- B phosphorylation, while high-concentration ABPS alleviates immune stress by suppressing TLR4 mRNA, NF- B mRNA, and NF- B protein expression.

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Note: Figure translations are in progress. See original paper for figures.