Anemonin improves intestinal barrier restoration and influences TGF-β1 and EGFR signaling pathways in LPS-challenged piglets

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Abstract

The present study was aimed at investigating whether dietary anemonin could alleviate LPS-induced intestinal injury and improve intestinal barrier restoration in a piglet model. Eighteen 35-d-old pigs were randomly assigned to three treatment groups (control, LPS and LPS+anemonin). The control and LPS groups were fed a basal diet, and the LPS + anemonin group received the basal diet + 100 mg anemonin/kg diet. After 21 d of feeding, the LPS- and anemonin-treated piglets received i.p. administration of LPS; the control group received saline. At 4 h post-injection, jejunum samples were collected. The results showed that supplemental anemonin increased villus height and transepithelial electrical resistance, and decreased crypt depth and paracellular flux of dextran (4 kDa) compared with the LPS group. Moreover, anemonin increased tight junction claudin-1, occludin and ZO-1 expression in the jejunal mucosa, compared with LPS group. Anemonin also decreased TNF-α, IL-6, IL-8 and IL-1β mRNA expression. Supplementation with anemonin also increased TGF-β1 mRNA and protein expression, Smad4 and Smad7 mRNA expressions, and epidermal growth factor and epidermal growth factor receptor (EGFR) mRNA expression in the jejunal mucosa. These findings suggest that dietary anemonin attenuates LPS-induced intestinal injury by improving mucosa restoration, alleviating intestinal inflammation and influencing TGF-β1 canonical Smads and EGFR signaling pathways.

Introduction

Pulsatilla chinensis is a traditional Chinese herb that has been used in China for the treatment of enteritis and bacillary dysentery for many years.[1,2] The important bioactive compounds are anemonin, protoanemonin and saponin, which have been shown to possess anti-inflammatory, antibacterial, antiviral and antitoxic properties.[3,4] Anemonin is thought to be the primary compound responsible for the multiple activities of the plant.[4–6] Recently, a growing body of evidence has indicated that anemonin can be effective in protection in a number of injury models.[7–9] It has also been reported that anemonin exerts a beneficial role in ameliorating mucosal damage by enhancing the repair in trinitrobenzene sulfonic acid-induced colitis in rats.[10] However, no research has been conducted on the beneficial effects of anemonin on intestine injury in weaned piglets.

After injury, mucosal repair is a complex event that is highly regulated by multiple growth factors in the damaged intestine.[11–13] TGF-β1 and epidermal growth factor receptor (EGFR) signaling pathway were suspected to play an important regulatory role in restoration of damaged intestine.[14–16] TGF-β is a multifunctional polypeptide growth factor that has a central role in modulating gut mucosal cell growth, differentiation, migration and epithelial restitution.[14,17] The TGF-β signaling pathway is mediated by the Smad

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family of proteins.[18] The role of TGF-β1 in the restoration of barrier integrity has been demonstrated in our previous studies.[16] Expression of epithelial growth factor (EGF) and EGFR were reported to correlate positively with the recovery of the small intestinal mucosa.[15,19,20] However, until now, few data are have been available on the e ects of anemonin on TGF-β1 and EGF, and their receptors and downstream signaling pathways in weaned pigs intestine after injury.

We hypothesized that anemonin may ameliorate LPS-induced intestinal injury and improve restoration by regulating the TGF-β1 and EGFR signaling pathways. In the present experiment, *Escherichia coli* LPS was administered to establish the model of gut injury. The purpose of the present study was to test this hypothesis and to elucidate the underlying mechanisms.

Materials and methods

Animals and treatment

The experiment was approved by the Animal Care and Use Committee, Zhejiang University. Eighteen 35-d-old pigs (Duroc×Landrace×Yorkshire, weaned at 21 d of age), with an average mass of 9.5 kg, were randomly assigned to three treatment groups, each consisting of six animals. One group served as the control group, while the other two groups were subjected to the induction of intestine injury by injection of LPS. Each group was treated with the following diets: (1) control group (piglets fed the control diet); (2) LPS group (piglets fed the control diet); (3) LPS + anemonin group (piglets fed the diet + 100 mg anemonin/kg diet). Anemonin [powder; purity ≥ 98% (Zhejiang Chinese Medical University, Hangzhou, China)] was well mixed with the basal diet. The dosage of 100 mg anemonin/kg diet was chosen because it was found in our preliminary study to be more e ective in improving morphology and function of the pig small intestine compared with other doses. Pigs were individually housed in pens $(1.8 \times 1.1 \, m^2)$ in an environmentally controlled nursery barn. The room temperature was maintained at 25–27°C. Each pen contained a feeder and a nipple waterer to allow piglets free access to feed and water. There were six replicate pens for each treatment. Diets were formulated to meet or exceed requirements suggested by the National Research Council (2012) (Table 1). After 21-d feeding with control or 100 mg anemonin/kg diet, the two challenged groups (LPS group and LPS + anemonin group) were injected i.p. with *E. coli* LPS (*E. coli* serotype 055: B5, Sigma Chemicals, St. Louis, MO, USA) at 100 μg/kg body mass (BM) and the unchallenged group was injected with the same amount of 0.9% NaCl solution. The dose of LPS was chosen to cause acute intestinal injury in weaned pig, in accordance with Liu et al.[21]

Table 1. Ingredients and chemical composition of the weaned diet on an as-fed basis.

Ingredients (g/kg)	Basal diet
Maize	573.5
Soybean meal	280
Fish meal	55
Dried whey	45
Limestone meal	5
Dicalcium phosphate	11.5
Sodium chloride	3
L-Lysine HCl	1.5
DL-Methionine	0.5
Vitamin-mineral premix[a]	10
Soybean oil	15
Composition (g/kg)	
Digestible energy (MJ/kg)[b]	14.40
Crude protein	229.6
Lysine	14.1
Methionine	4.6
Calcium	8.5
Total phosphorus	7.4

[a]Provided per kg of diet: vitamin A, 6000 IU; vitamin E, 50 IU; vitamin D_3, 600 IU; vitaminK_3, 1.5 mg; biotin, 0.10 mg; riboflavin, 8.0 mg; thiamine, 2.0 mg; niacin, 30 mg; pantothenic acid, 20 mg; pyridoxine, 3.0 mg; folic acid, 0.6 mg; vitamin B_{12}, 0.04 mg; choline, 800 mg; Cu (CuSO_4·5H_2O), 16 mg; Fe (FeSO_4), 125 mg; Zn (ZnSO_4), 100 mg; Mn (MnSO_4·H_2O), 15 mg; Se (Na_2SeO_3), 0.3 mg; I (KI), 0.2 mg.
[b]Digestible energy was calculated from data provide by Feed Database in China (2011).

Sample collection

Four h post-injection of LPS or saline (on d 21), piglets were killed under anesthesia with an i.v. injection of sodium pentobarbital (50 mg/kg BM) and the gastro-intestinal tract quickly removed. Segments of proximal jejunum were harvested immediately after killed and prepared for Ussing chamber studies. Adjacent specimens were fixed in 10% bu ered formalin until morphology measurements. Mucosal scrapings from the remaining jejunum were collected, rapidly frozen in liquid nitrogen and stored at –80°C.

Intestinal morphology and barrier function

After fixation, the intestinal segments were dehydrated, embedded in para n, sectioned into 5-μm samples and stained with hematoxylin and eosin.[22] Villus height and crypt depth were measured in three intestinal cross-sections per animal with at least 10 well-oriented crypt villus units for each cross-section using image analysis (Leica Imaging Systems, Wetzlar, Germany) and averaged for each sample. Transepithelial electrical resistance (TER) and mucosal-to-serosal permeability to 4-kDa FITC dextran (FD4; Sigma-Aldrich, St.

Louis, MO, USA) were determined *in vitro* in a Ussing chamber system, according to the procedures outlined by Moeser et al.[23] Briefly, segments of jejunum were stripped from the seromuscular layer in oxygenated (95% O_2/5% CO_2) Ringer's solution and then mounted in the Easy Mount Ussing chamber system with a multi-channel voltage-current clamp (model VCC MC6; Physiologic Instruments). The clamps were connected to acquire and analyze software (Physiologic Instruments, San Diego, CA, USA) for automatic data collection. After a 30-min equilibration period on the Ussing chambers, TER ($\Omega \cdot cm^2$) was recorded at 15-min intervals over a 2-h period and then averaged to derive the TER values for a given pig. FD4 (Sigma-Aldrich) was added on the mucosal side at a final concentration of 0.375 mg/ml. Mucosal-to-serosal flux of FD4 ($\mu g/cm^2/h^1$) was monitored from the serosal side at 30-min intervals for 120 min. The concentrations of FD4 in the serosal side were measured by fluorescence microplate reader (FLx800; Bio-Tek Instruments Inc., Winooski, VT, USA). The flux over the 2-h period was calculated.

Protein expression analysis by Western blot

The Western blot analysis was performed according to the procedures outlined by Hu et al.[22] Briefly, after electrophoresis the proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were incubated with primary Ab at 4°C for 10 h and then with the secondary Ab for 2 h at room temperature. The primary Abs [occludin, claduin-1, zonula occludens-1 (ZO-1), TGF-β1, β-actin] were purchased from Santa Cruz Technology Inc. (Santa Cruz, CA, USA). The secondary Ab was HRP-conjugated anti-rabbit Ab (Cell Signaling Technology, Danvers, MA, USA). Western blot was detected with an enhanced chemiluminescence detection kit (Amersham, Arlington Heights, IL, USA), photographed by a ChemiScope 3400 (Clinx Science Instruments, Shanghai, China) and analyzed using Quantity One software. β-Actin was used as an internal control, which exhibited no dierence among each group. The relative abundance of each target protein was expressed as target protein/β-actin protein ratio. The protein expression of all samples was expressed as fold changes, calculated relative to the control group.

mRNA expression analysis by RT-PCR

The mRNA levels of TNF-α, IL-1β, IL-6, IL-8, IFN-γ, TGF-β1 and TGF-β receptors, as well as their downstream signals Smads (Smad2, Smad3, Smad4 and Smad7), EGF and EGFR were determined by RT-PCR, as described by Liu et al.[21] Briefly, total RNA was extracted from jejunal mucosa using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), following

Table 2. Forward and reverse primers used for real-time PCR.

Target	Primer sequence
TNF-α	F:5′CATCGCCGTCTCCTACCA3′
	R:5′CCCAGATTCAGCAAAGTCCA3′
IL-6	F:5′CCTGTCCACTGGGCACATAAC3′
	R:5′CAAGAAACACCTGGCTCTGAAAC3′
IL-8	F:5′TGGCAGTTTTCCTGCTTTCT 3′
	R:5′CAGTGGGGTCCACTCTCAAT 3′
IL-1β	F:5′CAAAGGCCGCCAAGATATAA 3′
	R:5′GAAATTCAGGCAGCAACAT 3′
IFN-γ	F:5′GAGCCAAATTGTCTCCTTCTAC3′
	R:5′CGAAGTCATTCAGTTTCCCAG3′
TβRI	F:5′CTGTGTCTGTCCACCATTCATTTG3′
	R:5′CAACTTTGCTATGTCTGTCTCCCC3′
TβRII	F:5′CATCTCCTGCTAATGTTGTTGCC3′
	R:5′CGGTTCTAAATCCTGGGACACG3′
Smad2	F:5′GAAGAGAAGTGGTGTGAGAAAGCAG3′
	R:5′AATACTGGAGGCAAAACTGGTGTC3′
Smad3	F:5′TGGAGGAGGTGGAGAAATCAGAAC3′
	R:5′CACACTCGCTTGCTCACTGTAATC3′
Smad4	F:5′CCTGAGTATTGGTGTTCCATTGC3′
	R:5′TGATGCTCTGCCTTGGGTAATC3′
Smad7	F:5′TACTGGGAGGAGAAGACGAGAGTG3′
	R:5′TGGCTGACTTGATGAAGATGGG3′
EGF	F:5′TGCCATAAGGGTGTCAGGTATTT3′
	R:5′TGCTTTGCTCTTGCCCTCTAC3′
EGFR	F: 5′GGCCTCCATGCTTTTGAGAA 3′
	R:5′GACGCTATGTCCAGGCCAA3′
GAPDH	F:5′ATGGTGAAGGTCGGAGTGAAC3′
	R:5′CTCGCTCCTGGAAGATGGT3′

the manufacturer's guidelines. The purity and concentration of all RNA samples were measured using a Nano Drop spectrophotometer (ND-2000; NanoDrop Technologies, Wilmington, DE, USA). Reverse transcription using the PrimeScripte RT reagent kit (TaKaRa Biotechnology, Dalian, China) was carried out following the manufacturer's instructions. Quantitative analysis of PCR was carried out on a StepOne Plus real-time PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR Green Master mix (Promega, Madison, WI, USA), according to the manufacturer's instructions. The primers used are given in Table 2. Gene-specific amplification was determined by melting curve analysis and agarose gel electrophoresis. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative expression (fold changes), calculated relative to the values from the control group. The change (Δ) in Ct values in each group was compared with the Ct value of GAPDH (ΔCt). Subsequently, $\Delta\Delta$CT was computed for each target gene from the treatment groups by subtracting the averaged_ΔCT for the control group. The final fold

di erences were computed as $2^{-\Delta\Delta Ct}$ for each target gene. The results showed that GAPDH exhibited no di erence between two groups.

Statistical analysis

Data were analyzed using the SAS statistical package (SAS Institute, Cary, NC,USA), with each animal considered an experimental unit. Data were subjected to ANOVA. Di erences between the other means were tested using Duncan's multiple range tests. Di erences were considered significant at $P < 0.05$.

Results

Intestinal morphology and barrier function

The data for jejunal morphology and barrier function of piglets are shown in Table 3. Compared with the control group, the pigs challenged with LPS had shorter ($P < 0.05$) villus height, as well as reductions ($P < 0.05$) in the ratio of villus height to crypt depth in the jejunum. LPS challenge also increased the jejunal mucosal-to-serosal flux of FD4 flux and decreased ($P < 0.05$) the jejunal TER. In comparison with the LPS piglets, dietary supplementation of 100 mg anemonin/kg diet significantly increased ($P < 0.05$) villus height, ratio of villus height to crypt depth and TER, and decreased ($P < 0.05$) FD4 flux in the jejunum.

Tight junction protein expression

Figure 1 shows the tight junction protein levels of occludin, claudin-1 and (ZO-1 in the jejunal mucosa. As compared with the control group, LPS challenge decreased ($P < 0.05$) protein expressions of occludin, claudin-1 and ZO-1. However, dietary supplementation with 100 mg anemonin/kg diet resulted in an increase

Table 3. Effects of dietary anemonin on jejunum morphology and barrier function of piglets.

Item	Control	LPS	LPS + anemonin	SEM[3]
Villus height (μm)	381[a]	343.2[b]	368.5[a]	5.15
Crypt depth (μm)	111.33	121.57	117.92	3.22
Villus height:crypt depth	3.42[a]	2.84[c]	3.31[b]	0.06
TER ($\Omega \cdot cm^2$)	63.12[a]	46.35[b]	57.97[a]	2.98
FD$_4$ flux (μg/cm^{-2}/h^{-1})	1.21[c]	2.59[a]	1.54[b]	0.10

FD4: fluorescein isothiocyanate dextran (4 kDa).
[a,b,c]Means within a row with different letters differ significantly ($P < 0.05$). Data are means of six pigs.

($P < 0.05$) in protein expressions of occludin and claudin-1 compared with the LPS group.

Pro-inflammatory cytokine mRNA

The mRNA abundance of cytokines (TNF-α, IL-6, IFN-γ, IL-1β, and TGF-β1) in the jejunal mucosa of piglets is shown in Table 4. In comparison with the control group, piglets challenged with LPS had higher ($P < 0.05$) mRNA expressions of TNF-α, IL-1β, IL-6, IFN-γ and IL-8 in the jejunal mucosa, while LPS had no e ect on TGF-β1 mRNA ($P > 0.05$). Dietary addition of 100 mg anemonin/kg diet decreased ($P < 0.05$) the TNF-α, IL-1β, IL-6 and IL-8 levels, and increased the mRNA abundance of TGF-β1 compared with the LPS group.

TGF-β1 expression

Figure 2 presents the protein expression of TGF-β1 in the jejunal mucosa of piglets. Piglets challenged with LPS did not di er ($P > 0.05$) from the control group with regard to TGF-β1 protein expression. Dietary supplementation with 100 mg anemonin/kg diet significantly increased ($P < 0.05$) the expression of TGF-β1 compared with the LPS and control groups. Consistent with the increased protein expression of TGF-β1, Table 4 shows that 100 mg anemonin/kg diet supplementation also significantly improved ($P < 0.05$) the mRNA expression of TGF-β1.

mRNA expression of Smads

Table 5 shows mRNA expression of TGF-β receptor (TβRI and TβRII) in the jejunal mucosa of piglets. TβRI and TβRII mRNA expressions did not di er ($P > 0.05$) between control and LPS-treated piglets. Dietary supplementation with 100 mg anemonin/kg diet exhibited an increase ($P < 0.05$) in gene expression of TβRII. The mRNA abundance of Smads components (Smad2, Smad3, Smad4 and Smad7) in the jejunal mucosa is shown in Table 5. Compared with the control group, the pigs challenged with LPS did not di er ($P > 0.05$) from the control group with regard to Smad mRNA expression. However, dietary supplementation with 100 mg anemonin/kg diet resulted in an increase ($P < 0.05$) in mRNA expression of Smad4 and Smad7.

mRNA expression of EGFR signal pathways

Table 6 shows mRNA expressions of EGF and EGFR in the jejunal mucosa of piglets. In comparison with the control group, LPS challenge resulted in a decrease ($P < 0.05$) in mRNA expression of EGFR mRNA in the jejunal mucosa of piglets. No di erence in EGF mRNA levels were detected ($P > 0.05$) among the

control group and LPS group. However, 100 mg ane-monin/kg diet supplementation significantly increased ($P < 0.05$) EGF and EGFR mRNA expression in jejunal mucosa compared with the LPS group.
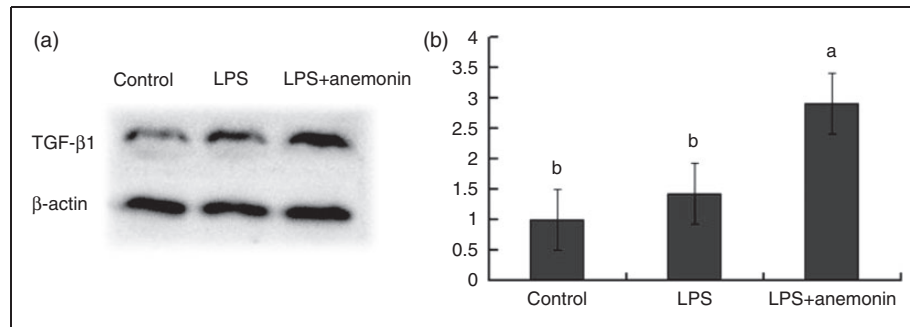
Figure 2. Effects of dietary anemonin on the protein expression of TGF-β1 in the jejunal mucosa of piglets. (a) Representative blots of TGF-β1 expression and β-actin. (b) Relative TGF-β1 protein expression in jejunal mucosa of piglets.
a,bMean values with different letters were significantly different ($P < 0.05$). Values are means and SD, represented by vertical bars. The control sample was used as the reference sample. The protein expression of all samples was expressed as fold changes, calculated relative to the control group. Control (non-challenged control), piglets receiving a control diet and injected with 0.9% sterile saline; LPS (LPS-challenged control), piglets receiving the same control diet and injected with *E. coli* LPS; LPS + anemonin (LPS challenged + 100 mg/kg anemonin), piglets receiving a 100 mg/kg anemonin diet and injected with LPS.

Table 5. Effects of dietary anemonin mRNA expressions of Smad signals on the jejunal mucosa of piglets.[a]

Item	Control	LPS	LPS + anemonin	SEM
TβRI	1.00	1.22	1.13	0.19
TβRII	1.00[c]	1.42[c]	2.81[b]	0.28
Smad2	1.00	1.14	1.52	0.24
Smad3	1.00	1.75	1.23	0.28
Smad4	1.00[c]	1.57[c]	3.74[b]	0.37
Smad7	1.00[c]	1.36[c]	4.33[b]	0.38

[a]The $2^{-\triangle\triangle Ct}$ method was used to analyze the relative expression (fold changes), calculated relative to the values in samples from the control pigs. Data are means of six pigs.
b,cWithin a row with different letters differ significantly ($P < 0.05$)

Table 6. Effects of dietary anemonin on EGF and EGFR mRNA expression in the jejunal mucosa of piglets.[a]

Item	Control	LPS	LPS + anemonin	SEM[2]
EGF	1.00[c]	1.25[c]	2.69[b]	0.29
EGFR	1.00[b]	0.57[c]	1.36[b]	0.13

[a]The $2^{-\triangle\triangle Ct}$ method was used to analyze the relative expression (fold changes), calculated relative to the values in samples from the control pigs. Data are means of six pigs.
b,cMeans within a row with different letters differ significantly ($P < 0.05$).

villus height:crypt depth ratio after LPS challenge, which indicated that supplementation improved intestinal morphology after damage. Our data were supported by the results of Xu et al.,[29] who reported anemonin alleviated mouse intestinal ultrastructure after exposure to PRV and *E. coli* infection. Liu et al.[30] also reported that Merbal medicine (Chinese *Pulsatilla* root) improved mucosa structure by increasing villus height and decreasing crypt depths in the

small intestine of canine. Consistent with improved intestinal morphology, the present study also demonstrated that anemonin improved intestinal barrier function, reflected by increased TER and decreased FD4 flux in jejunum of piglets compared with the LPS group. These findings support the notion that anemonin beneficially ameliorates the LPS-induced damage in intestinal structure and function. According to these results, we speculated that enhancing the restoration of epithelium after injury might be the mechanism by which anemonin protects intestinal integrity and barrier function in an LPS-induced injury model.

The intestinal barrier is mainly formed by a layer of epithelial cells joined together by tight junctions.[31] Of them, occludin, claudins and ZO-1 are thought of as the major integral membrane proteins, which participate in tight junction structural integrity by binding to the actin cytoskeleton.[32] LPS has been reported to disrupt barrier function in a time- and dose-dependent manner, and also induces a redistribution of tight junction proteins (occludin, claudin-1and ZO-1) from the intercellular junctions.[28,33] In the current experiment, our results convincingly indicated that LPS reduced the abundance of occludin, claudin-1 and ZO-1 in the small intestinal mucosa of piglets. Consistent with the improved intestinal morphology and barrier function, it is noteworthy that anemonin supplementation increased the protein expression of occludin, claudin-1 and ZO-1 in the small intestine of LPS-challenged piglets. Similarly, Zhang and Jiang reported that *Pulsatilla* alcohol extract protected intestinal epithelial tight junctions in rats with trinitrobenzene sulfonic acid-induced colitis.[10] Consequently, in the present study, anemonin may have partially improved the intestinal integrity and barrier function via increasing tight junction protein expression.

Cytokines also play an important role in the regulation of the intestinal barrier integrity.[34] Overproduction

of pro-inflammatory cytokines has a negative influence on gut integrity and epithelial function.[35,36] In the present study, pro-inflammatory cytokine (TNF-α, IL-1β, IL-6, IFN-γ and IL-8) gene expression was elevated in the jejunal mucosa of piglets challenged with LPS. Consistent with improved intestinal integrity, dietary anemonin decreased cytokine expression (TNF-α, IL-6, IL-1β, IL-8). Currently, there is an abundance of research on the modulation of intestinal pro-inflammatory mediators through supplementation of anemonin.[5,21] In line with our findings, Sang et al. reported that the protective role of *Pulsatilla* extract in LPS-induced inflammatory responses is partially due to its inhibitory effects on the overproduction of pro-inflammatory mediators, including pro-inflammatory cytokines (TNF-α, IL-1β, IL-6).[8] Therefore, in the current study, feeding pigs with an anemonin-supplemented diet may improve intestinal integrity, partially by inhibiting the production of pro-inflammatory mediators.

TGF-β is considered to be the key regulator of intestinal injury restoration as most of the cytokines that promote epithelial restitution act through a TGF-β-dependent mechanism.[16] It has been reported to play an important role in the post-weaning adaptation process in the intestine of pigs.[14,16] In the present study, we found that anemonin increased the TGF-β1 content in the jejunum mucosa of piglets, both in gene and protein expression, compared with the LPS-challenged pigs. Similarly, Liu et al.[7] found that the anemonin from *P. chinensis* prevents diarrhea in mice by regulating TGF-β1 expression. Increased TGF-β1 due to the traditional medicine shikonin was also reported to regulate positively wound healing after injury.[37] It is likely that the increased TGF-β1 concentration induced by anemonin was directly responsible for the restoration of the intestine barrier function. Therefore, we hypothesize that the beneficial role of anemonin in intestinal barrier protection may be partially influenced by TGF-β1.

The canonical TGF-β signaling pathway is mediated by the Smad family of proteins.[18] Smad proteins—the only substrates of TβR kinase—are critical mediators of the TGF-β signaling transducer. When TGF-β reaches the membrane of target cells, it binds directly to TGF-β type II receptors (TβRII), which leads to the recruitment of TGF-β type I receptors (TβRI). TβRII then trans-phosphorylates TβRI, enabling the TβRI kinase domain to act on cytoplasmic proteins and thereby propel downstream signaling actions. Smad2 and Smad3 are receptor-regulated Smads (R-Smads). Following stimulation by TGF-β, Smad2 and Smad3 become phosphorylated. Phosphorylated Smad 2/3 can complex with Smad4 (the common Smad mediator), and then translocate to the nucleus and regulate gene expression.[38] To determine whether the TGF-β1 signaling pathway is involved in anemonin exerting beneficial

effects on LPS-induced intestinal injury, we evaluated the canonical downstream substrates of the TGF-β signal (TβRI, TβRII, Smad2, Smad3, Smad4 and Smad7) and observed that anemonin supplementation increased the gene expressions of TβRII, Smad4 and Smad7 after LPS challenge, indicating that the canonical Smads signaling pathway was activated. However, mRNA expression of Smad2 and Smad3 in the intestinal mucosa did not vary significantly compared with the LPS and control groups. In line with our study, another study has shown that TGF-β1 protects the immature gut from injury via Smad protein-dependent signaling pathways.[39] Consequently, we speculate that anemonin-improved intestinal barrier restoration in LPS-induced intestine injury may partially be induced through the TGF-β1 Smad pathway, either directly or indirectly.

EGF/EGFR signaling also plays an important role in the repair of the small intestinal mucosa following damage.[13,15] It is crucial for promoting enterocyte proliferation and restitution, as well as regeneration of the mucosal epithelium.[15,40,41] Accumulating evidence has shown that the EGF/EGFR signal improves healing of the gastrointestinal tract and enhances gut integrity and intestinal barrier function.[42–44] EGF also possesses protective barrier effects on intestinal epithelia and has often been related to effects on cell proliferation and/or epithelial restitution, which could indirectly or secondarily affect the tight junction.[45] In the present study, an interesting finding was that dietary anemonin supplementation increased the gene expression of EGFR in the jejunum mucosa, which indicates that anemonin can activate EGFR signaling in protecting the intestinal barrier after LPS challenge. This is consistent with our observations that anemonin treatment resulted in higher villus height and lower villus height:crypt depth ratio in the jejunum of LPS-challenged piglets. Moreover, we observed that anemonin significantly increased EGF mRNA levels in the jejunum mucosa of the LPS-challenged piglets. Similarly, Liu et al.[7] demonstrated that anemonin improved the restoration of intestinal mucosa in diarrheal mice by increased expression of TGF-β1 and EGFR. Therefore, it is possible that the effects of anemonin on intestinal integrity recovery are also related to activation of EGFR signaling.

In conclusion, dietary supplementation with an 100-mg anemonin/kg diet is beneficial for alleviating intestine injury and improving mucosal repair in piglets. Possible mechanisms for the actions of anemonin may include the following: (1) reduction of pro-inflammatory cytokines (indicated by decreased TNF-α, IL-1β, IL-6 and IL-8); (2) alleviation of intestinal injury (indicated by alleviation of LPS-induced damage in the intestinal structure and barrier function, and increased expression of tight junction protein levels of occludin and claudin-1 in the jejunal mucosa of the

LPS-challenged piglets); and (3) improvement of mucosal repair via TGF-β1 canonical Smad pathways and EGFR signaling (indicated by increase in TGF-β1 and EGFR expression in the jejunm mucosa). These findings may have important implications for treating intestinal damage in humans and other animals.

Declaration of Conflicting Interests

Funding

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