Original Article

Innate Immunity

Innate Immunity
2016, Vol. 22(5) 344–352

DOI: 10.1177/1753425916648223
ini.sagepub.com
SAGE

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.

Keywords
Anemonin, intestinal injury, TGF-β1, EGFR, piglets

Date received: 10 January 2016; revised: 30 March 2016; accepted: 14 April 2016

Introduction

Pulsatilla chinensis C

C

.[1,2] T

.[3,4] A

.[4–6] R

.[7–9] I

.[10] H

A

.[11–13] TGF-β1
(EGFR)

.[14–16] TGF-β

.[14,17]

T TGF-β S

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.[18] T TGF-β1 -

.[16] E
(EGF) EGFR

.[15,19,20] H , ,

TGF-β1 EGF, -

W
LPS-
TGF-β1 EGFR -
. I , *Escherichia coli* LPS

T

Materials and methods

Animals and treatment

T A C
U C , Z U . E 35- -
(D ×L ×Y ,
21), 9.5 , -
, -
. O
,
LPS.
E : (1) -
( ); (2) LPS
( ); (3) LPS +
(+ 100 /).
A [; ≥98% (Z C
M U , H , C)]
. T 100 /

. P
(1.8 × 1.1 2)
. T 25
27°C. E
. T
. D -

N R C (2012) (T 1). A 21-
100 / ,
(LPS LPS +
) . . *E. coli* LPS (*E. coli*
055: B5, S C , S . L , MO, USA)
100 µ / (BM)
0.9% N C -
. T LPS
, L .[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 ($CuSO_4{\cdot}5H_2O$), 16 mg; Fe ($FeSO_4$), 125 mg; Zn ($ZnSO_4$), 100 mg; Mn ($MnSO_4{\cdot}H_2O$), 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

F - LPS (21),
. .
(50 / BM) -
. S
U . A -
10% -
. M
,
80°C.

Intestinal morphology and barrier function

A , ,
, 5-µ
.[22] V
-
10 -
-
(L I S , W , G)
. T
(TER) - -
4- D FITC (FD4; S -A , S .

L , MO, USA) *in vitro* U M .[23] B , (95% O_2/5% CO_2) R E M U - - (VCC MC6; P I). T - (P I , S D , CA, USA) . A 30- U , TER (Ω· [2]) 15- 2- TER . FD4 (S - A) - 0.375 / . M - - FD4 (μ / [2]/ [1]) 30- 120 . T FD4 (FL 800; B -T [redacted] I I ., W [redacted], VT, USA). T 2- .

Protein expression analysis by Western blot

T W H .[22] B , - - (M , B , MA, USA). T A 4°C 10 A 2 . T A [, -1, -1 (ZO-1), TGF-β1, β-] S C T I . (S C , CA, USA). T A HRP- - - A (C S T , D , MA, USA). W [redacted] (A , A H , IL, USA), - C S 3400 (C S I , S , C) Q O . β-A , . T /β- . T ,

mRNA expression analysis by RT-PCR

T RNA TNF-α, IL-1β, IL-6, IL-8, IFN-γ, TGF-β1 TGF-β , - S (S 2, S 3, S 4 S 7), EGF EGFR RT-PCR, L .[21] B , RNA TRI (I , C , CA, USA),

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′

' . T - RNA N D (ND-2000; N D T , W , DE, USA). R - P S RT [redacted] (T K R B , D , C) ' . Q PCR S O P - PCR (A B , F C , CA, USA) SYBR G M (P , M , WI, USA), ' . T - T 2. G - . T $2^{-\Delta\Delta C}$ ( ), . T (Δ) C C GAPDH (ΔC). S , ΔΔCT ΔCT . T

2 ΔΔC
. T GAPDH
.

Statistical analysis

D SAS
(SAS I , C , NC,USA),
. D
ANOVA. D
D ' . D
$P < 0.05$.

Results

Intestinal morphology and barrier function

T
T 3. C
, LPS
($P < 0.05$) ,
($P < 0.05$)
. LPS
FD4
($P < 0.05$) TER. I
LPS , 100
/ ($P < 0.05$)
, TER,
($P < 0.05$) FD4 .

Tight junction protein expression

F 1
, -1 (ZO-1 .
A , LPS
($P < 0.05$) ,
-1 ZO-1. H ,
100 /

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 (Ω·cm²)	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.

($P < 0.05$)
-1 LPS .

Pro-inflammatory cytokine mRNA

T RNA (TNF-α, IL-6,
IFN-γ, IL-1β, TGF-β1)
T 4. I
, LPS
($P < 0.05$) RNA TNF-α, IL-1β, IL-6,
IFN-γ IL-8 , LPS
TGF-β1 RNA ($P > 0.05$). D
100 / ($P < 0.05$)
TNF-α, IL-1β, IL-6 IL-8 ,
RNA TGF-β1
LPS .

TGF-β1 expression

F 2 TGF-β1
. P
LPS ($P > 0.05$)
TGF-β1 . D
100 /
($P < 0.05$) TGF-β1
LPS .
C
TGF-β1, T 4 100 /
($P < 0.05$) RNA TGF-β1.

mRNA expression of Smads

T 5 RNA TGF-β
(TβRI TβRII) .
TβRI TβRII RNA
($P > 0.05$) LPS- .
D 100 /
($P < 0.05$)
TβRII. T RNA S
(S 2, S 3, S 4 S 7)
T 5. C
, LPS
($P > 0.05$)
S RNA . H ,
100 /
($P < 0.05$) RNA
S 4 S 7.

mRNA expression of EGFR signal pathways

T 6 RNA EGF EGFR
. I
, LPS
($P < 0.05$) RNA EGFR RNA
. N EGF
RNA ($P > 0.05$)

LPS . H , 100 -

/

($P < 0.05$) EGF EGFR RNA _ -

LPS .

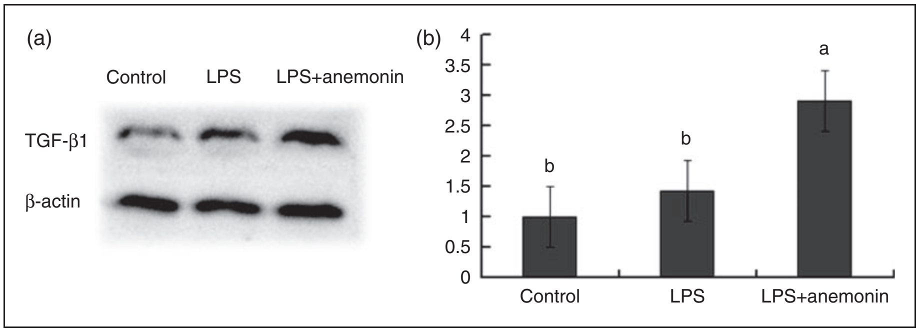

Figure 2. Effects of dietary anemonin on the protein expression of TGF-β1 in the jejunal mucosa of piglets. (a) Representative blots of TGF-β1 expression and β-actin. (b) Relative TGF-β1 protein expression in jejunal mucosa of piglets.
[a,b]Mean values with different letters were significantly different ($P < 0.05$). 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 $2^{-\Delta\Delta Ct}$ 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,c]Within 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 $2^{-\Delta\Delta Ct}$ 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,c]Means within a row with different letters differ significantly ($P < 0.05$).

: LPS ,
. O
X .,[29]
PRV *E. coli* . L
.[30] M (C
Pulsatilla)

. C
,
, TER FD4
LPS
. T
LPS-
. A
,
LPS- .
T
.[31] O
, , ZO-1
,
.[32] LPS
,
(, -1 ZO-1)
.[28,33] I ,
LPS
, -1 ZO-1
. C
,
, -1
ZO-1 LPS-
. S , Z J *Pulsatilla*
.[10] C , ,
(C .
C
.[34] O

-
.[35,36] I -
, - (TNF-α, IL-1β, IL-6, IFN-γ IL-8)
LPS.
C ,
(TNF-α, IL-6, IL-1β, IL-8). C ,
- -
-
.[5,21] I , S .
Pulsatilla LPS-
-
, -
(TNF-α, IL-1β, IL-6).[8] T , -
- -
,
,
-
.
TGF-β -
TGF-β-
.[16] I
- -
.[14,16] I ,
TGF-β1
,
, LPS- .
S , L .[7]
P. chinensis
TGF-β1 . I TGF-β1 -
.[37] I
TGF-β1 -
. T , -
TGF-β1.
T TGF-β
S .[18] S
TβR
TGF-β . W TGF-β
,
TGF-β II (TβRII),
TGF-β I (TβRI). TβRII
- TβRI, TβRI
. S 2
S 3 - S (R-S).
F TGF-β, S 2 S 3
. P S 2/3
S 4 (S),
.[38] T TGF-β1 -

LPS- ,
TGF-β
(TβRI, TβRII, S 2, S 3, S 4
S 7)
TβRII, S 4
S 7 LPS ,
S .
H , RNA S 2 S 3
-
LPS . I
, TGF-β1 -
S -
.[39] C ,
-
LPS- -
TGF-β1 S ,
.
EGF/EGFR
.[13,15] I -
,
.[15,40,41] A
EGF/EGFR
.[42–44] EGF -
/
, -
.[45] I ,
-
EGFR
,
EGFR
LPS . T
:
LPS- .
M ,
EGF RNA
LPS- . S , L .[7]
TGF-β1 EGFR. T , -
EGFR
.
I ,
100- /
-
. P
: (1) - -
(TNF-α,
IL-1β, IL-6 IL-8); (2)
(LPS-
,
-1

LPS-); (3) -
TGF-β1 S
EGFR (TGF-β1
EGFR). T -
-
.

Declaration of Conflicting Interests

T ( )
, , /
.

Funding

T ( ) -
, , /
: T N
N S F C (31472103),
S F A R P
I (201403047).

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