Berberine inhibits lipopolysaccharide-induced expression of inflammatory cytokines by suppressing TLR4-mediated NF-κB and MAPK signaling pathways in rumen epithelial cells of Holstein calves

Chenxu Zhao, Yazhou Wang, Xue Yuan, Guoquan Sun, Bingyu Shen, Feng Xu, Guyue Fan, Meiyu Jin, Xinwei Li, Guowen Liu

SUPPLEMENTARY FILE

Supplementary Materials and Methods

The Ethics Committee on the Use and Care of Animals at Jilin University approved the study protocol (Changchun, China).All animals use in this study were approved by the Chinese Society of Laboratory Animal Sciences (2015 clinical trial [2015-121]).

Rumen epithelial cell culture

The method of Sun et al (2017) was used. Ruminal epithelia were collected from three healthy, newborn (24-hour-old), fasting female Holstein calves weighing 35-45 kg. The calves were euthanized by thiamylal sodium under sterile conditions and a portion of the ruminal epithelium was excised. Epithelia were placed into plates filled with cold phosphate-buffered saline (PBS) and immediately washed with sterile water. The tissues were cut into small pieces (3 to 4 cm^2, approximately 20 g) and placed into 250 mL bottles containing 100 mL of a penicillin and streptomycin solution at concentrations of 2500 U/mL and 2500 mg/mL, respectively (Sun *et al.* 2017). The bottles were transferred to a slow-shaking orbital hot air incubator at 37°C for 30 min (Klotz *et al.* 2001). Next, the tissues were transferred to new 250 mL bottles containing 100 mL of a gentamicin and amphotericin B at concentrations of 1000 U/mL and 12 μg/mL, respectively, and placed in a shaking orbital hot air incubator at 37°C for 30 min. The rumen mucosae were stripped down with ophthalmic scissors and tweezers and then minced into 1 mm^3 pieces and washed twice with PBS. Cells were separated using a digestion solution containing 0.25% trypsin. Approximately 20 g of tissue was added to 40 mL of digestion solution and incubated in an incubator at 37°C. After tryptic digestion for 10-15 min, the solution was filtered with 100 mesh (150 μm) cell sieves. Cells were isolated using 5 to 7 cycles of digestion with trypsin (15-20 min per cycle) depending on the degree of digestion that was required for the mucosa. The second, third, and fourth cycles of cell suspensions were collected in sterile 500 mL beakers. An equal volume of Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum (FBS) (HyClone, Logan, UT) was added to terminate the tryptic digestion. Then, the cell suspensions were centrifuged for 10 min at 1000 rpm. The sediments were washed with PBS, and centrifuged for 10 min at 1000 rpm. The cells were resuspended with DMEM, and filtered with 300 mesh (37.5

μm) cell sieves. The trypan blue dye exclusion method was used to assess cell viability. The cell density was adjusted to 5×10^4, 1×10^5, or 1×10^6 cells/mL. The cell suspensions were seeded sequentially into 96-well tissue culture plates (100 μL/well) for cck8 assay. The cell suspensions seeded into 24-well plates and covered by glass coverslips was 1 mL/well, while 6-well tissue culture plates received 2 mL/well and were incubated at 37°C in 5% CO_2. The medium composed of DMEM containing 15% FBS, 6 μg/mL gentamicin, 6 μg/mL amphotericin B, 200 mg/mL streptomycin and 200 U/mL penicillin was replaced every 24 h.

CCK-8 assay

Cell counting kit-8 (CCK-8) is used for simple and accurate cell proliferation and toxicity analysis. REC was inoculated in 96-well culture plates and adjusted to 1×10^5 cells/mL. There were 100 μL cells in each well culturing for 12 h. The cell-free supernatants were discarded. After incubation with BBR (10, 25, 50, 70, or 100 μM, 50 μL/well; Sigma-Aldrich, MO, USA) for 1 h, the cells were stimulated by LPS (4 μg/mL, 50 μL/well; Sigma-Aldrich, MO, USA) for 18 h. CCK8 was added into all of the experimental groups (10 μL/well; Sigma-Aldrich, MO, USA) and incubated for 3 h. The OD value was measured at 450nm using a microplate reader (Thermo Scientific Instrument Inc., Shanghai, China).

Enzyme-linked immunosorbent assay (ELISA)

REC were treated with different concentrations of BBR (50, 70, or 100 μM) and 4 μg/mL LPS in 24-well plates (1×10^6 cells/mL). Cell-free supernatants were subsequently centrifuged at 3000 rpm for 20 min to extract the liquid in the upper layer for using in assays for the inflammatory cytokines TNF-α, IL-1β, and IL-6 by ELISA kits (TNF-α: ml024586, 6.3-8000 pg/ml; IL-6: ml023756, 1-200 pg/ml; IL-1β: ml023753, 1-640 pg/ml; Shanghai Enzyme-linked Biotechnology Co., Ltd., Shanghai, China) according to the manufacturer's instructions, respectively.

RNA extraction and real-time PCR

Total RNA was extracted with TRIzol reagent (TaKaRa Biotechnology Co., Ltd., Tokyo, Japan) according to the supplier's protocol. The concentration and purity of RNA was determined by K5500 Micro-Spectrophotometer (Beijing Kaiao Technology Development Co., Ltd., Beijing, China). Then, 5 μg of total RNA was reverse-transcribed into cDNA using a reverse transcription kit (TaKaRa Biotechnology Co., Ltd.). The mRNA expression levels were evaluated by quantitative polymerase chain reaction (qRT-PCR) analysis using a SYBR Green QuantiTect RT-PCR Kit (Takara Biotechnology Co., Ltd.). As shown in Table 1, primers were designed using Primer 5.0 (Canada Premier Company). The PCR reactions were carried out as the following: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Gene expression levels were analyzed with the $2^{-\Delta\Delta CT}$ method, where CT is the cycle threshold (Rao *et al.* 2013).

Western blotting

The cell medium was discarded and PBS was used to wash the surface of cells. The cells were scraped into the 1.5mL tubes and centrifuged at 800 rpm for 10 min. The total cellular proteins were extracted using a protein extraction kit (Sangon Biotech Co., Ltd., Shanghai, China). The concentration of protein was assayed using BCA protein assay kit according to the manufacturer's instructions (Beyotime Biotechnology Inc., Shanghai, China). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) separation was performed with 40 μg of protein per lane and with known molecular weight markers (Sangon Biotech Co., Ltd.). Then we transferred the target protein onto polyvinylidene fluoride (PVDF) membrane. Then, the membranes were blocked with 5% nonfat dry milk in Tris buffered saline-Tween (TBST) for 4 h using a mechanical horizontal rotator bed at room temperature. The blocked membranes were hybridized overnight at 4°C with antibodies against NF-κB p-p65, TLR4, MyD88 (ab86299, ab22048, ab2068, Abcam, Cambridge, MA, USA), NF-κB p65, IκBα, p-IκBα, JNK, p-JNK, ERK, p-ERK, p38MAPK, p-p38MAPK (4764, 4814, 9246, 9252, 9251, 4695, 4370, 8690, 4511, Cell Signaling Technology, Danvers, MA, USA) and β-actin (sc-47778,Santa Cruz, CA, USA) respectively. Then the membranes were washed three times with TBST for 5 min each time with gentle agitation and incubated with peroxidase-conjugated secondary antibody (1:5,000 dilutions in TBST) at room temperature for 45 min. The final blots were developed using enhanced chemiluminescence solution (Pierce Biotechnology Inc., Chicago, IL, USA) in a Western blotting detection system (ProteinSimple, Santa Clara, CA, USA).

Immunocytofluorescence

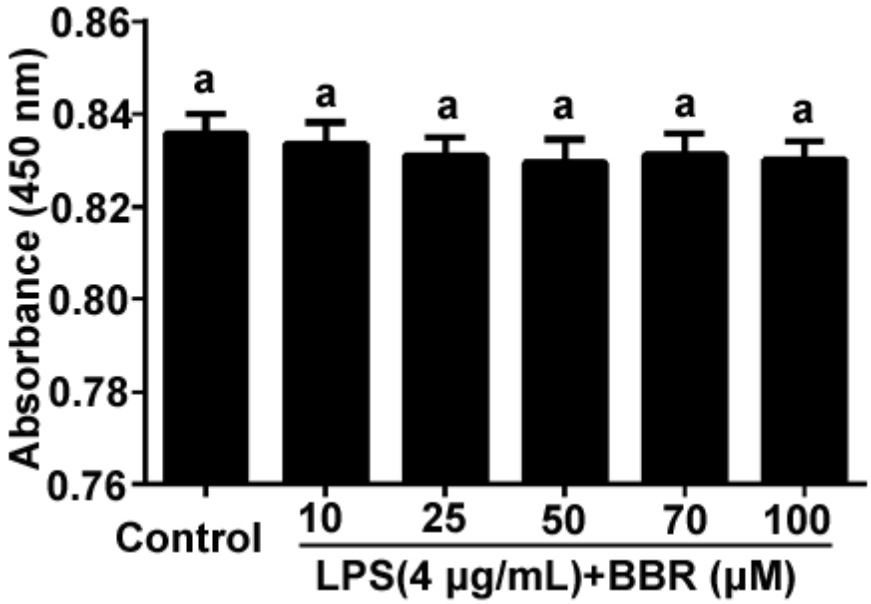
Cells were cultured on glass coverslips at a density of 5×10^4 cells/well in a 24-well culture plate. They were grown to approximately 90% confluency and treated with 100 μM BBR and 4 μg/mL LPS. The coverslips were then washed three times with PBS, fixed with a 4% paraformaldehyde/PBS for 20 min at room temperature, washed three times with PBS, and then subjected to antigen retrieval with EDTA-Na_2 (95°C, 5 min), after which they were rewashed. Following treatment with 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO) and washing with PBS three times, the cells were blocked with a goat serum-containing blocking buffer. After further washes, the slides were incubated with primary antibody against NF-κB p65 overnight at 4°C and then exposed to goat anti-rabbit IgG conjugated with Cy3 (Beyotime Biotechnology Inc.) for 30 min. and counterstained with Hoechst 33258 (Beyotime Biotechnology Inc.). Each step included three washes with PBS. Immunofluorescence microscopy was performed using a confocal laser microscope (FluoView FV1200, Olympus, Tokyo, Japan).

Supplementary Table S1 The primers sequences of the genes

Gene	Primer sequences (5'-3')	Tm（℃）	Length
TNFα	For CTGCCGGACTACCTGGACTAT	60.75	234 bp
	Rev CCTCACTTCCCTACATCCCTAA	58.35	
IL-6	For AACGAGTGGGTAAAGAACGC	58.49	140 bp
	Rev CTGACCAGAGGAGGGAATGC	59.82	234
IL-1β	For CTGAACCCATCAACGAAA	52.48	190 bp
	Rev ATGACCGACACCACCTGC	59.65	
β-actin	For GCCCTGAGGCTCTCTTCCA	60.99	101bp
	Rev GCGGATGTCGACGTCACA	60.13	

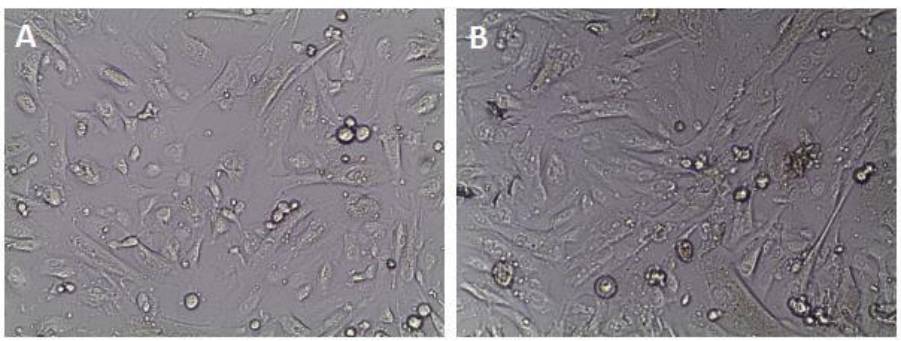
Supplementary Figure S1

Effects of different concentrations of BBR on the cell viability. Cells were cultured with BBR (10, 25, 50, 70, or 100 μM) and LPS (4μg/mL). The data are presented as the mean ± SEM. a: the same letter indicates the lack of significant difference (P > 0.05).



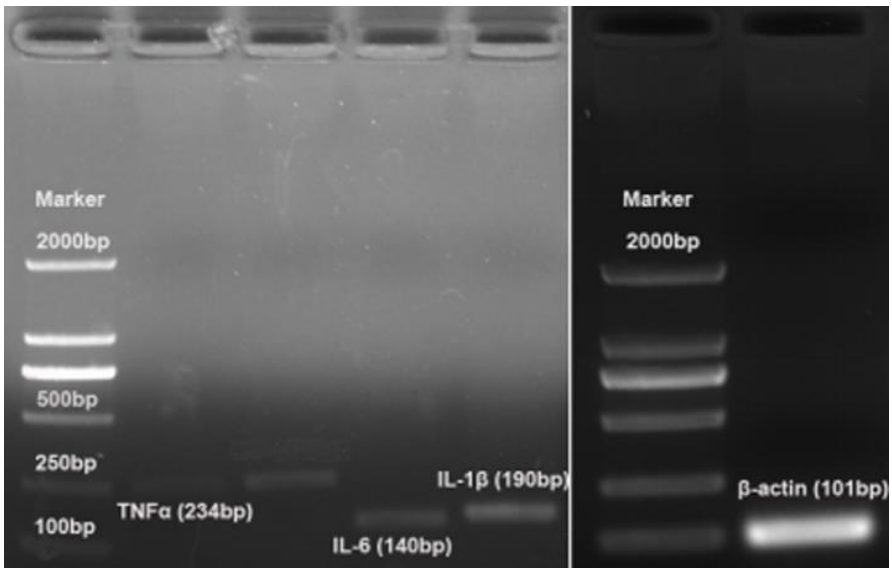
Supplementary Figure S2

The morphology of rumen epithelial cells was observed under microscope(100×).(A) REC wastreated withnothing as a control. (B) REC wastreated withBBR(100 *μM*). There is no marked difference inappearance betweenthe REC treated and untreated with BBR.



Supplementary Figure S3

The agarose gel electrophoresis of the PCR products of TNF-α, IL-1β,IL-6andβ-actin. The products were all single bands without any stray bands.



Supplementary Figure S4

The Melt Curve of qPCR products ofTNF-α, IL-1β,IL-6andβ-actin. (A) TNF-α.(B) IL-1β.(C) IL-6.(D) β-actin. The products were all single crests without any stray crests.