Journal of Dairy Research

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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

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Research Article

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Cite this article: Zhao C *et al* (2019). 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. *Journal of Dairy Research* **86**, 171–176. https://doi.org/10.1017/S0022029919000323

Received: 14 September 2018
Revised: 27 January 2019
Accepted: 6 February 2019
First published online: 30 May 2019

Keywords:
Berberine; MAPK; NF-κB; rumen epithelial cell

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Abstract

Subacute ruminal acidosis (SARA) can increase the level of inflammation and induce rumenitis in dairy cows. Berberine (BBR) is the major active component of *Rhizoma Coptidis*, which is a type of Chinese anti-inflammatory drug for gastrointestinal diseases. The purpose of this study was to investigate the anti-inflammatory effects of BBR on lipopolysaccharide (LPS)-stimulated rumen epithelial cells (REC) and the underlying molecular mechanisms. REC were cultured and stimulated with LPS in the presence or absence of different concentrations of BBR. The results showed that cell viability was not affected by BBR. Moreover, BBR markedly decreased the concentrations and mRNA expression of pro-inflammatory cytokines, including tumor necrosis factor-α, interleukin-1β, and interleukin-6 in the LPS-treated REC in a dose-dependent manner. Importantly, Western blotting analysis showed that BBR significantly suppressed the protein expression of toll-like receptor 4 (TLR4) and myeloid differentiation primary response protein (MyD88) and the phosphorylation of nuclear factor-κB (NF-κB), inhibitory kappa B (IκBα), p38 mitogen-activated protein kinase (MAPK), and c-Jun N-terminal kinase (JNK) in LPS-treated REC. Furthermore, the results of immunocytofluorescence showed that BBR significantly inhibited the nuclear translocation of NF-κB p65 induced by LPS treatment. In conclusion, the protective effects of BBR on LPS-induced inflammatory responses in REC may be due to its ability to suppress the TLR4-mediated NF-κB and MAPK signaling pathways. These findings suggest that BBR can be used as an anti-inflammatory drug to treat inflammation induced by SARA.

Subacute ruminal acidosis (SARA) is a nutrition-associated metabolic disease with high incidence rate in dairy cows. Studies in the United States have indicated that up to 19% of early-lactation dairy cows and 26% of mid-lactation cows have SARA (Enemark, 2008). Highly fermentable diets are often fed to high producing lactating cows to increase milk production, which can reduce ruminal pH, microbial activity and increase the risk of SARA (Pan *et al*., 2016). Dairy cows with SARA display ruminal inflammation (rumenitis), which is associated with high a concentration of lipopolysaccharide (LPS) in the rumen (Gozho *et al*., 2007; Dionissopoulos, 2013). LPS interacts with a class of receptors known as toll-like receptor 4 (TLR4) and activates various inflammatory pathways mediated by mitogen-activated protein kinases (MAPK) and nuclear factor kappa B (NF-κB) (Nyati *et al*., 2017), which promote the expression of pro-inflammatory mediators including the cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6) (Di *et al*., 2011; Chen *et al*., 2015). Wellnitz and Kerr (2004) have reported that LPS interacts with epithelial cells and stimulates the production of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, which is further involved in the induction of inflammation (Wellnitz and Kerr, 2004). Zhang *et al*. (2016) also reported that LPS stimulation can induce high levels of mRNA expression of various cytokines in the rumen epithelial cells of Holstein cows, especially the IL-1β. Therefore, the rumenitis may be a direct result of high level of ruminal LPS induced by SARA.

Berberine (BBR) is the main component of the Chinese medicine *Rhizoma Coptidis*, which is often used to treat inflammation of the gastrointestinal tract in human (Chen *et al*., 2014). It has been reported that BBR suppresses the expression of IL-1β in LPS-treated THP-1 cells (Zhou *et al*., 2017). Furthermore, Guo *et al*. (2016) also proved that BBR ameliorated liver inflammation and decreased hepatic IL-1β and TNF-α mRNA levels (Guo *et al*., 2016).

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Berberine is widely used in veterinary clinics in China. Zhou *et al.* (2015) have reported that berberine could prevent and treat multi-drug resistant *S. epidermidis* mastitis in dairy cows (Zhou *et al.*, 2015). However, whilst an anti-inflammatory role for BBR is generally accepted, little detail of the mechanism has been revealed, and BBR has not previously been studied in LPS-stimulated bovine ruminal epithelial cells (REC). Our hypothesis was that BBR would have an anti-inflammatory effect on LPS-stimulated REC, and in addition we wished to understand the underlying mechanism. The results of this study will lay a foundation for the possible application of BBR on the prevention and treatment of rumenitis induced by SARA.

Material 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 to isolate and prepare rumen epithelial cells for culture. Briefly, ruminal epithelia were collected under terminal anaesthesia from newborn female Holstein calves weighing 35–45 kg. Washed epithelia were minced and digested with trypsin. After centrifugation, cell suspensions were resuspended in Dulbecco's modified Eagle's medium (DMEM) containing 15% fetal bovine serum (FBS) (HyClone, Logan, UT) 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. Full details of the cell preparation and culture are given in the online Supplementary File.

CCK-8 assay

Cell counting kit-8 (CCK-8) is used for simple and accurate cell proliferation and toxicity analysis. REC were added to 96-well culture plates and adjusted to 1×10^5 cells/ml. There were 100 μl cells in each well which were cultured 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 450 nm 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

Standard methods were used to isolate total RNA for reverse-transcription into cDNA. The mRNA expression levels were evaluated by quantitative polymerase chain reaction (qRT-PCR) analysis using SYBR Green and gene expression levels were analyzed with the $2 - \Delta\Delta CT$ method, where CT is the cycle threshold (Rao *et al.*, 2013). Details are given in the online Supplementary File, including the primers used (online Supplementary Table S1). Primers were designed using Primer 5.0 (Canada Premier Company), the gel electrophoresis data are in Supplementary Fig. S3 and the melt curve analysis is shown at Supplementary Fig. S4.

Western blotting

Standard Western blotting techniques were used and full details are given in the online Supplementary File. 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.). Target proteins were transferred to polyvinylidene fluoride (PVDF) membrane and blocked with 5% nonfat dry milk. 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 and incubated with peroxidase-conjugated secondary antibody (1:5000 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).

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Statistical analysis

All data were analyzed with SPSS (Statistical Package for the Social Sciences) 16.0 software (SPSS Incorporated, Chicago, IL, USA). The data with more than two groups were tested with ANOVA with subsequent Bonferroni correction and expressed as the mean ± standard error of mean (SEM). $P < 0.05$ was considered to be statistically significant.

Results

Effects of BBR on cell viability

The potential cytotoxicity of BBR (in absence or presence of LPS) on REC was analyzed by CCK-8 assay. Cell viabilities were not affected by BBR (10, 25, 50, 70, and 100 μM) and LPS (4 μg/ml) (online Supplementary Fig. S1). The morphology of treated and non-treated REC did not differ (Supplementary Fig. S2).

Effects of BBR on the Expression of Inflammatory Cytokines

The levels of pro-inflammatory cytokines were measured using ELISA kits. LPS stimulated the expected increase in the contents of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 (Fig. 1A, C and E). Importantly, BBR treatment significantly decreased the concentrations of TNF-α, IL-1β, and IL-6 in LPS-treated REC in a dose-dependent manner (Fig. 1A, C and E). To further investigate the effect of BBR on the pro-inflammatory cytokines, the mRNA expression levels of the cytokines were measured by qRT-PCR. The results showed that BBR treatment significantly decreased the mRNA expression of TNF-α, IL-6, and IL-1β in LPS-stimulated REC (Fig. 1B, D and F). Overall, these results indicate that BBR can significantly decrease the synthesis of pro-inflammatory factors (TNF-α, IL-1β, and IL-6) in LPS-treated REC.

BBR suppresses TLR4-mediated NF-κB and MAPK pathways

TLR4 and MyD88 are upstream molecular targets of NF-κB and MAPK and can directly regulate the activation of these pathways in LPS-induced inflammation (Nyati *et al.*, 2017). Our results showed that BBR down-regulated the expression of TLR4 and MyD88 in LPS-treated REC in a dose-dependent manner (Fig. 2).

The NF-κB and MAPK pathways play important roles in the induction of inflammatory cytokines. We, therefore, investigated the effects of BBR on the NF-κB and MAPK pathways in LPS-treated REC. As shown in Figs. 3 and 4, LPS treatment over-activated the NF-κB and MAPK inflammatory pathways. The phosphorylation levels of p65 and IκBα in the NF-κB pathway and the phosphorylation levels of p38MAPK, ERK, and JNK in the MAPK pathway were markedly increased. In contrast, BBR (50, 70, and 100 μM) inhibited the phosphorylation of IκBα and p65, which indicated that BBR treatment inhibited the over-activation of the NF-κB signaling pathway induced by LPS in REC. Furthermore, BBR treatment could significantly inhibit the phosphorylation of JNK and p38MAPK. However, there was no significant effect on the phosphorylation of ERK. These results indicate that BBR treatment can significantly decrease the activation of NF-κB- and MAPK-mediated inflammatory pathways in LPS-treated REC.

To further investigate the effect of BBR on the NF-κB pathway, the nuclear localization of NF-κB p65 was detected by immunocytofluorescence. NF-κB p65 was translocated to the nucleus of REC after LPS treatment, whereas BBR (100 μM) treatment partially prevented this phenomenon (Fig. 5), which further demonstrated that BBR attenuated the activation of LPS-induced NF-κB inflammatory pathway in REC.

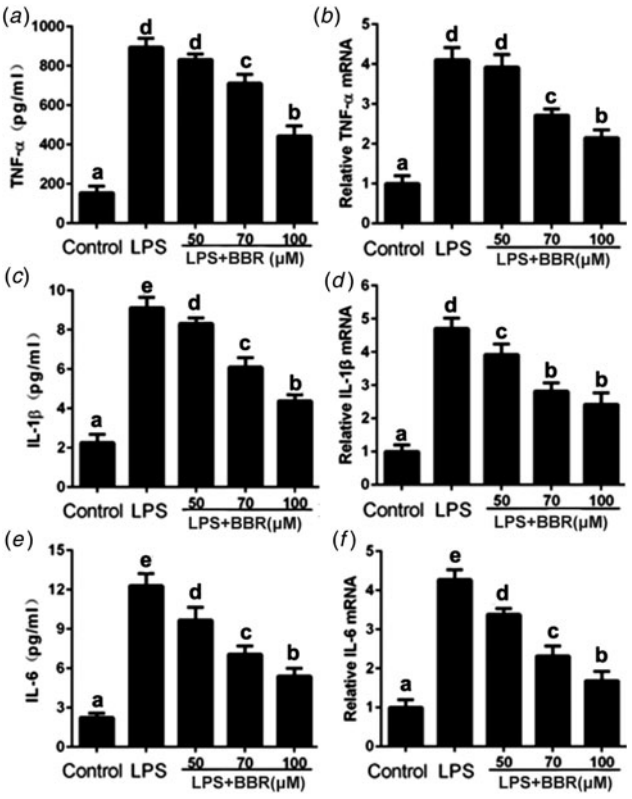

Fig. 1. Effects of different concentrations of BBR on the production of inflammatory mediators. REC was treated with different concentrations of BBR (50, 70, or 100 μM) and 4 μg/ml LPS in 24-well plates (1×10^6 cells/ml). The levels of TNF-α, IL-1β and IL-6 in the LPS-induced inflammation in the rumen epithelial cells were measured by ELISA. (A) The concentration of TNF-α. (B) The mRNA level of TNF-α. (C) The concentration of IL-1β. (D) The mRNA level of IL-1β. (E) The concentration of IL-6. (F) The mRNA level of IL-6. Similar results were obtained from three independent experiments. The data are presented as the mean ± SEM ($n = 6$ in each group). a, b, c, and d: the same letter indicates the lack of significant difference ($P > 0.05$), whereas different letters indicate significant difference ($P < 0.05$).

Discussion

SARA is a common disease in high-yielding dairy cows that are fed highly digestible diets. A reduction in ruminal pH below 5.6 for more than 3 h per day is characteristic of SARA (Gozho *et al.*, 2005). During low ruminal pH, gram-negative bacteria are lysed more rapidly, increasing the concentration of LPS in the rumen (Khafipour *et al.*, 2011). It has been reported that intramammary infections caused by *E. coli* are more easily identified because they are characterized by a severe inflammatory response, including sudden shock, sepsis, and often death, caused by the releasing of LPS (Moretti *et al.*, 2017). The ruminal epithelium is continuously exposed to high level of LPS in cows with SARA. LPS is an inflammatory inducer and is involved in the development of rumenitis. Zhang *et al.* (2016) also reported that LPS is involved in the development of inflammation in cows with SARA (Zhang *et al.*, 2016). BBR has been reported to have an anti-inflammatory effect (Jeong *et al.*, 2009; Zha *et al.*, 2010). In the present study, we investigated the anti-inflammatory effects and mechanism of BBR on LPS-stimulated

https://doi.org/10.1017/S0022029919000323 Published online by Cambridge University Press

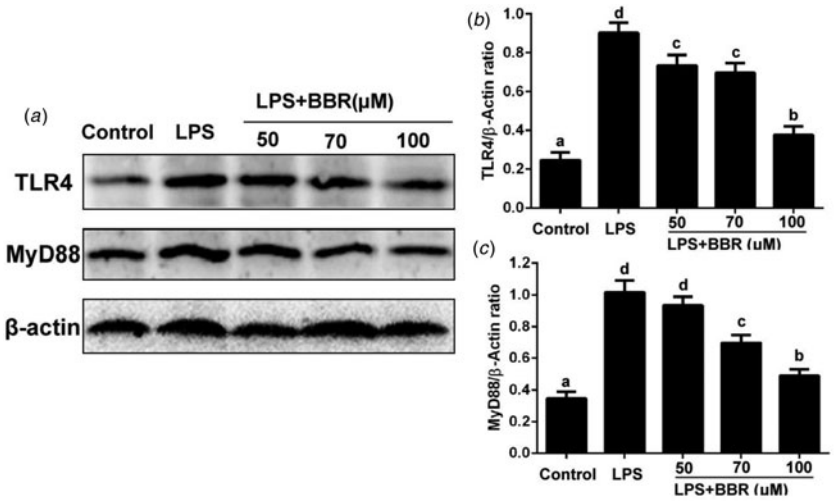

Fig. 2. Effects of BBR on the protein expression of TLR4 and MyD88. REC was treated with different concentrations of BBR (50, 70, or 100 μM) and 4 μg/ml LPS in 6-well plates (1×10^6 cells/ml). Protein samples were analyzed by Western blotting with specific antibodies; β-actin was used as a control. (A) The Western blotting results for TLR4 and MyD88. (B) The protein level of TLR4. (C) The protein level of MyD88. Similar results were obtained from three independent experiments. The data are presented as the mean ± SEM ($n = 6$ in each group). a, b, c, and d: the same letter indicates the lack of significant difference ($P > 0.05$), whereas different letters indicate significant difference ($P < 0.05$).

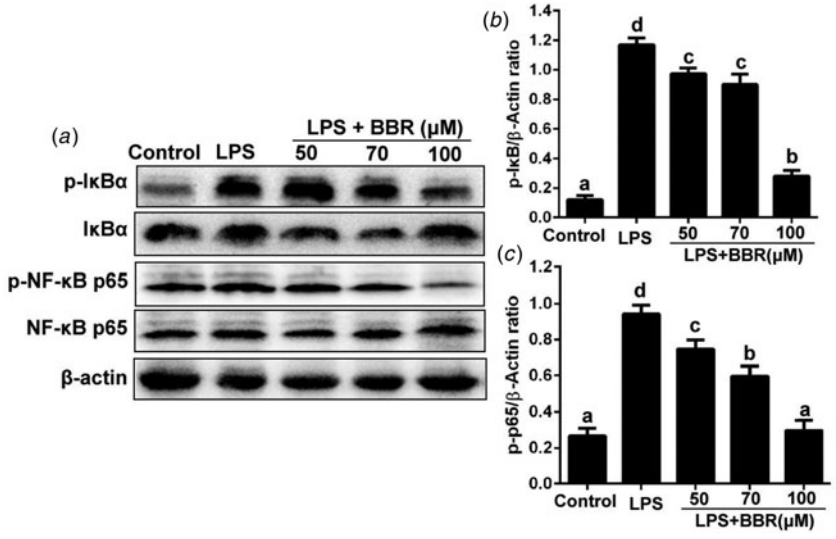

Fig. 3. Effects of BBR on NF-κB activation. REC was treated as described in Fig. 2. Protein samples were analyzed by Western blotting with specific antibodies; β-actin was used as a control; p indicates phosphorylation. Similar results were obtained from three independent experiments. The data are presented as the mean ± SEM ($n = 6$ in each group). a, b, c, and d: the same letter indicates the lack of significant difference ($P > 0.05$), whereas different letters indicate significant difference ($P < 0.05$).

REC. Our results showed that BBR suppressed the expression of pro-inflammatory cytokines by preventing TLR4-mediated NF-κB and MAPK activation in LPS-stimulated REC, indicating that the anti-inflammatory mechanism of BBR was associated with suppressing TLR4-mediated NF-κB and MAPK signaling pathways.

Rumenitis is the direct result of SARA in dairy cows. Janssens and Beyaert (2003) reported that LPS could cause inflammatory damage of ruminal epithelial cells in dairy cows with forestomach atony (Janssens and Beyaert, 2003). Importantly, Zhang *et al.* (2016) found that LPS treatment could significantly increase the mRNA expression of IL-1β and IL-8 in ruminal epithelial cells of Holstein cows *in vitro* (Zhang *et al.*, 2016). These studies indicated that the ruminal epithelial cells are capable of responding to the release of inflammatory cytokines. Thus, we used ruminal epithelial cells to investigate the anti-inflammatory mechanisms of BBR *in vitro*. Our results showed that LPS significantly increased the expression and production of inflammatory cytokines TNF-α, IL-6, and IL-1β in ruminal epithelial cells. These further mediated the damage caused by inflammation in the ruminal epithelium. Interestingly, BBR treatment significantly decreased the overproduction of inflammatory cytokines induced by LPS in a dose-dependent manner, which indicated that BBR could improve LPS-induced inflammation in the ruminal epithelium.

TLR4, one of the best characterized TLRs, is a pattern recognition receptor for LPS (Li *et al.*, 2016; Wang *et al.*, 2018). TLR4 activates MyD88 and then induces the activation of NF-κB and MAPK signaling pathways to increase the release of pro-inflammatory cytokines (Lu *et al.*, 2008). The increasing LPS could activate TLR4 on the surface of mammary epithelial cells, resulting in up-regulated DNA binding activity of nuclear factor (NF)-κB and increased expression of inflammatory cytokines. It would act synergistically to disrupt mammary epithelial function, and cause inflammation in the whole body (Wang *et al.*, 2018). In this study, Western blotting results showed that BBR attenuated LPS-induced TLR4 and MyD88 expression in REC, which indicated that BBR could inhibit the LPS-induced TLR4 pathway. NF-κB p65 is a nuclear transcription factor that exists in an inactive form in the cytoplasm bound to its inhibitor IκB (Shi *et al.*, 2014). Once cells are treated with various inducers, IκB is degraded, and NF-κB p65 translocates into the nucleus after phosphorylation (Xiong *et al.*, 2015). Eventually, this promotes the transcription of target genes, such as TNF-α, IL-6,

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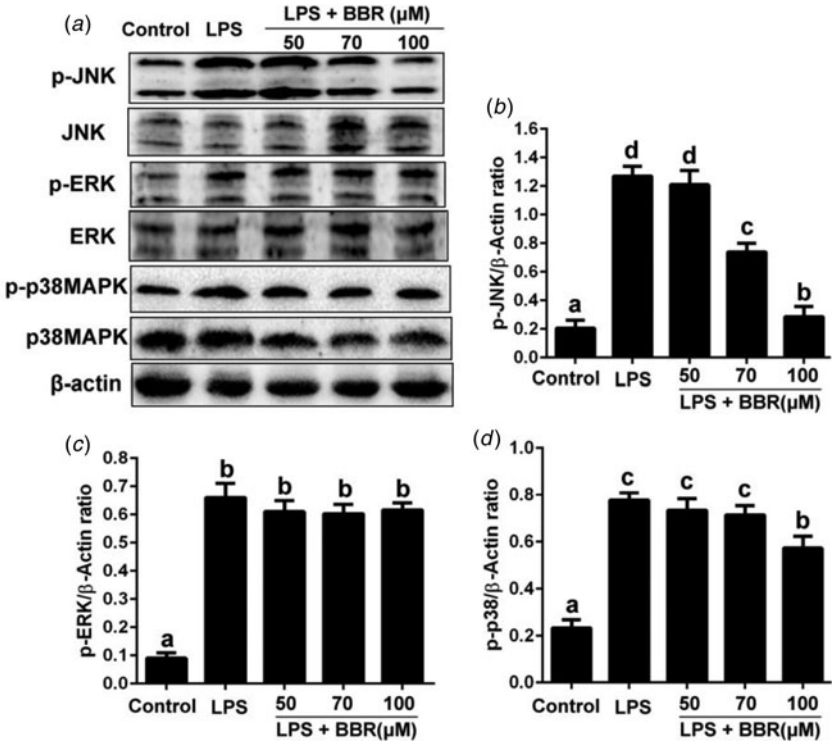

Fig. 4. Effects of BBR on the MAPK pathway. REC was treated as described in Fig. 2. Protein samples were analyzed by Western blotting with specific antibodies; β-actin was used as a control; p indicates phosphorylation. (A) The Western blotting results for JNK, p-JNK, ERK, p-ERK, p38MAPK, and p-p38MAPK. (B) The protein level of p-JNK. (C) The protein level of p-ERK. (D) The protein level of p-p38MAPK. Similar results were obtained from three independent experiments. The data are presented as the mean ± SEM ($n = 6$ in each group). a, b, c, and d: the same letter indicates the lack of significant difference ($P > 0.05$), whereas different letters indicate significant difference ($P < 0.05$).

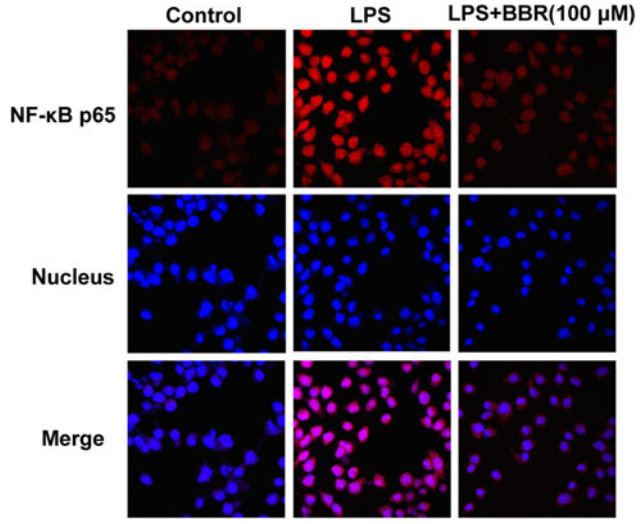

Fig. 5. Effects of BBR on the transcriptional activity and immunofluorescence staining of NF-κB p65 in REC. REC was treated with 4 μg/ml LPS and 100 μM BBR. Cellular localization of p65 was analyzed by immunofluorescence using laser confocal microscopy (400×).

and IL-1β (Shi *et al.*, 2014). To further characterize the inhibitory mechanism of BBR in cytokine production, we examined the effect of BBR on the NF-κB pathway. In this study, we found that exposure of ruminal epithelial cells to LPS could increase phosphorylation of IκBα and NF-κB p65 and promote the translocation of NF-κB p65 to the nucleus. When cells were treated with different concentrations of BBR, the phosphorylation of IκBα and NF-κB p65 was inhibited in a dose-dependent manner. These results suggested that BBR suppressed the production of pro-inflammatory cytokines by preventing the activation of NF-κB pathway in ruminal epithelial cells. Furthermore, over-activation of MAPK-mediated inflammatory pathway also plays a key role during inflammatory responses (Di *et al.*, 2011). We also examined the effect of BBR on the activation of p38, ERK and JNK in the MAPK pathway. Our results showed that BBR significantly inhibited the phosphorylation of JNK and p38MAPK but not that of ERK, which suggested that BBR could significantly inhibit the over-activation of JNK and p38MAPK induced by LPS. Taken together, our results clearly demonstrated that BBR suppressed pro-inflammatory cytokine production by preventing the activation of TLR4-mediated NF-κB and MAPK pathways induced by LPS in ruminal epithelial cells.

In conclusion, this study demonstrates that the anti-inflammatory effect of BBR in LPS-induced inflammation in ruminal epithelial cells may be due to its ability to inhibit the TLR4-mediated NF-κB and MAPK signaling pathways, which further inhibits the expression of TNF-α, IL-1β, and IL-6. Therefore, BBR may be a promising therapeutic reagent for rumen inflammation in cows with SARA.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029919000323

Acknowledgements. This work was supported by the National key research and development program (Beijing, China; grant no. 2016YFD0501206), the National Natural Science Foundation of China (Beijing, China; grant no. 31460681, 31472247, 31672621 and 31772810), the Jilin province education department project (Jilin, China; grant no. 440020032059).

Conflict of interest. The authors declare no conflicts of interest.

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