Sodium butyrate reduces bovine mammary epithelial cell inflammatory responses induced by exogenous lipopolysaccharide, by inactivating NF- κ B signaling

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ABSTRACT

Exogenous molecules derived from catabolic states (e.g., fatty acids, β -hydroxybutyrate) during periods of stress such as the periparturient period or pathogen challenges [e.g., lipopolysaccharide (LPS)] can trigger an inflammatory response in tissues such as the liver and the mammary gland. Butyrate is one of the major short-chain fatty acids produced in the rumen, and work with non-ruminants has demonstrated that it can alter inflammatory processes. The primary objective of this study was to explore the preventive effect of sodium butyrate (SB) on LPS-induced inflammation in bovine mammary epithelial cells along with underlying molecular mechanisms. Immortalized bovine mammary epithelial cells (MAC-T) were treated with SB (0.1, 0.25, 0.5, 1, 2, or 5 mM) or with the histone deacetylase inhibitor trichostatin A (TSA; 6.25, 12.5, 25, or 50 nM) for 18 h, followed by a challenge with 1 μ g/mL LPS for an additional 6 h. Pretreatment with SB prevented increase in apoptosis of LPS-challenged MAC-T cells in a dose-dependent manner. The LPS treatment up-regulated mRNA abundance of tumor necrosis factor α (*TNFA*), interleukin-6 (*IL6*), and interleukin-1B (*IL1B*), whereas inhibition of histone deacetylase with TSA dampened this effect. More importantly, SB had clear dose-dependent effects on the inflammatory response by preventing upregulation of *TNFA*, *IL6*, and *IL1B*. Furthermore, pretreatment with TSA or SB attenuated the downregulation of histone H3 acetylation protein abundance induced by LPS. The greater ratio of p-I κ B α /I κ B α and p-p65/p65 protein abundance and the increase in nuclear localization of NF- κ B p65 protein in response to LPS were attenuated by pretreatment with SB. Overall, the data indicated that exogenous SB

alleviates mammary cell pro-inflammatory responses partly through post-translational mechanisms that diminish NF- κ B signaling. Thus, the cytoprotective effect of SB against an inflammatory challenge might represent a preventive tool to help the mammary gland against pathogens such as those causing mastitis.

Key words: butyrate, inflammation, mastitis, NF- κ B pathway

INTRODUCTION

Mastitis is a widespread infectious disease in dairy herds worldwide (Yang et al., 2018). This disease is characterized by an inflammatory response in the mammary gland, resulting in reduced milk yield and quality (Wilson et al., 1997). Issues such as high cost of treatment and widespread epidemics not only have an economic impact but seriously affect the welfare and health of cows (Melchior et al., 2006; Viguier et al., 2009). Bovine mammary epithelial cells (BMEC) are not only essential for milk component synthesis but are also a major line of defense against the invasion of pathogens (Swanson et al., 2009). Both exogenous LPS from intramammary *Escherichia coli* and diet-induced endogenous LPS release from the rumen are considered to be major causative factors inducing BMEC inflammation (Fox, 2009).

Toll-like receptor 4 (TLR4) is an important pattern-recognition receptor that plays a crucial role in the innate immune system, which can be activated by LPS (Mukherjee et al., 2016). Upon activation by LPS, TLR4 stimulates downstream signaling pathways and triggers the production of inflammatory cytokines (Günther et al., 2017; Wang et al., 2018). Nuclear factor-kappa B (NF- κ B), which acts as a key inflammation regulator during bovine mastitis, is a downstream signaling molecule of TLR4 (Li et al., 2019); NF- κ B is normally maintained in an inactive form by sequestration in the cytoplasm through interaction with inhibitor of κ B α

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($\text{I}\kappa\text{B}\alpha$). A variety of extracellular stimuli lead to the rapid phosphorylation, ubiquitination, and ultimately proteolytic degradation of $\text{I}\kappa\text{B}\alpha$, which frees NF- κB to translocate to the nucleus, where it regulates transcription of tumor necrosis factor α (*TNFA*), interleukin-6 (*IL6*), and interleukin-1B (*IL1B*; Karin and Ben-Neriah, 2000). In turn, these cytokines further aggravate the pathology and inflammatory responses involved in mastitis. Because inactivation of the TLR4-NF- κB inflammation signaling pathway may protect BMEC against LPS-induced inflammation (Sun et al., 2019a), developing potential strategies to inhibit this pathway might lead to therapeutic approaches against mastitis.

Butyrate, one of the major short-chain fatty acids produced in the rumen, is a natural product of microbial fermentation of carbohydrate (Wong et al., 2006). Research with non-ruminant cell lines clearly underscored the anti-inflammatory, antioxidant and antiapoptotic properties of exogenous butyrate (Aguilar et al., 2014). Suppression of pro-inflammatory *TNFA*, *IL6*, and *IL1B* is one of the main responses induced by butyrate (Bailón et al., 2010), but it also could encompass alterations in the degradation or phosphorylation of $\text{I}\kappa\text{B}\alpha$ and dampening of NF- κB signaling (Machado et al., 2012; Lee et al., 2017). Recent studies with ruminal tissue (Dai et al., 2017) or bovine hepatocytes have underscored the anti-inflammatory potential of sodium butyrate (**SB**) at the transcriptional and post-translational level; for instance, SB inhibited histone deacetylase (**HDAC**) to control both abundance of pro-inflammatory cytokines and activity of NF- κB (Xu et al., 2018).

On the basis of available data, we hypothesized that SB might also protect against inflammatory responses induced by LPS in BMEC. Thus, the main objective of this study was to investigate the anti-inflammatory role of SB on BMEC by evaluating transcriptional and post-translational responses in vitro.

MATERIALS AND METHODS

Cell Culture and Treatment

The MAC-T cells were cultured in Dulbecco's modified Eagle medium, nutrient mixture F-12 (DMEM/F-12, Thermo Fisher Scientific, Grand Island, NY) containing 10% (vol/vol) fetal bovine serum (Thermo Fisher Scientific), 1% (vol/vol) antibiotic-antimycotic mix (penicillin 100 U/mL, streptomycin 100 $\mu\text{g}/\text{mL}$, and amphotericin B 0.25 $\mu\text{g}/\text{mL}$; Hyclone, Logan, UT) at 37°C in a humidified atmosphere of 5% CO_2 . The medium was changed every 24 h. The SB (303410; Sigma-Aldrich, St. Louis, MO) was dissolved in distilled H_2O , and trichostatin A (**TSA**) (647925; Sigma-Aldrich) was dissolved in ethanol. Cells were pretreated with SB (0.1,

0.25, 0.5, 1, 2, or 5 mM) for 18 h, and then treated with 1 or 20 $\mu\text{g}/\text{mL}$ LPS for 6 h in DMEM/F-12 medium without serum (to avoid fetal bovine serum affecting pro-inflammatory cytokine transcript abundance). Furthermore, cells were pretreated with TSA (6.25, 12.5, 25, or 50 nM) or ethanol [0.1% (vol/vol), vehicle control, **Veh**] in amounts equal to that present in TSA cultures for 18 h, and then treated with 1 $\mu\text{g}/\text{mL}$ LPS for 6 h in DMEM/F-12 medium without serum.

Cell Viability

Cell viability was measured using the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to manufacturer's instructions. The Cell Counting Kit-8 contains tetrazolium salt 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium monosodium salt (WST-8) and the intermediate electron carrier 1-methoxy-5-methylphenazinium methyl sulfate (mPMS). Adjacent to metabolically active cells, the pink WST-8 is reduced extracellularly to a yellow reaction product called formazan. Its formation can be quantified photometrically, with the amount of formazan generated being directly proportional to metabolic activity—hence, the number of living cells in a given biological sample. The MAC-T cells (1×10^5 cells/mL) were plated in 96-well plates at 37°C for 4 h. Cells were subsequently treated with 0.1, 0.25, 0.5, 1, 2, or 5 mM SB for 18 h, followed by a challenge with 1 or 20 $\mu\text{g}/\text{mL}$ LPS for 6 h. After treatment, cells were incubated with 10 μL of Cell Counting Kit-8 solution at 37°C for 2 h. The absorbance was measured at 450 nm with a microplate reader (Thermo Fisher Scientific). Cell viability was calculated as percentage relative to control cells, exposed to neither SB nor LPS.

Measurement of Cell Apoptosis

Cell apoptosis was determined according to a previous study (Sun et al., 2019b). After incubation with SB or LPS as indicated, cells were washed twice with PBS. Cell apoptosis was detected using an annexin V-FITC apoptosis detection kit (BD Pharmingen, San Jose, CA) and analyzed by flow cytometry (Becton Dickinson, San Jose, CA). Apoptotic cells were expressed as percentage of the total cells.

Immunofluorescence Staining

Immunofluorescence staining was performed as described by Xu et al. (2018). The MAC-T cells were seeded in laser confocal Petri dishes (Corning Life Science, Lowell, MA) and incubated with SB or LPS as indicated. Cells were washed 3 times with PBS and

fixed in 10% formaldehyde neutral buffer solution for 30 min at room temperature. After washing 3 times with PBS, antigen retrieval was performed using EDTA-Na at 95°C for 5 min. Cells were then permeabilized with 0.1% Triton X-100 (T9284; Sigma-Aldrich) for 10 min, followed by washing 3 times with PBS. Subsequently, cells were incubated with primary antibody for NF- κ B p65 (sc-8008; Santa Cruz Biotechnology, Santa Cruz, CA; 1:50) at 4°C overnight, followed by washing 3 times with PBS. Cells were incubated with goat anti-mouse IgG conjugated with cy3 (A0521; Beyotime Institute of Biotechnology, Shanghai, China), washed 3 times with PBS, and stained with 4',6-diamidino-2-phenylindole (DAPI, 1 μ g/mL; D8417, Sigma-Aldrich) for 10 min. Lastly, cells were visualized with a confocal laser microscope (TCS SP5, Leica Microsystems, Wetzlar, Germany), and images were taken under an Olympus (Tokyo, Japan) FLUOVIEW FV1000 microscope.

Total RNA Extraction and Quantitative Reverse-Transcription PCR

We performed RNA extraction using RNAiso Plus (Takara Biotechnology Co. Ltd., Dalian, China) following the supplier's protocols. Then, a K5500 Micro-Spectrophotometer (Beijing Kaiiao Technology Development Ltd., Beijing, China) was used to measure RNA purity and concentration, with RNA quality measured via electrophoresis (1% agarose gels).

In our study, the optical density (OD)₂₆₀/OD₂₈₀ ratio of the total RNA was determined to be 1.9, and the intensity of the 28S ribosomal RNA band was approximately twice the intensity of the 18S ribosomal RNA band in total RNA samples, indicating that total RNA was of high quality. One μ g total RNA was reverse-transcribed into cDNA using a reverse transcription kit (RR047A, Takara Biotechnology Co. Ltd.) according to manufacturer's instructions. The quantitative reverse-transcription PCR assays were performed as described by Zhu et al. (2019) using SYBR green plus reagent kit (Roche, Basel, Switzerland) with the 7500 Real-Time PCR System (Applied Biosystems, Waltham, MA). Primer sets are listed in Supplemental Table S1 (<https://doi.org/10.3168/jds.2020-18189>). Ubiquitin B and GAPDH, already known to be suitable for BMEC (Griesbeck-Zilch et al., 2008), were used to normalize target gene abundance, followed by calculation of abundance using the $2^{-\Delta\Delta CT}$ method. All reactions were run in triplicate.

Protein Extraction and Western Blotting

Cells were washed twice with 4°C PBS and extracted using a commercial protein extraction kit (C510003;

Sangon Biotech Co. Ltd., Shanghai, China) following the supplier's protocols. The BCA protein assays (P1511; Applygen Technologies, Beijing, China) were performed to measure protein concentration. Western blotting was performed as described previously (Sun et al., 2019b). Briefly, equal amounts of protein (30 μ g) were separated on an 8 to 15% Tris-glycine gel and electrotransferred onto 0.45 μ m polyvinylidene difluoride (PVDF) membranes. The PVDF membranes were blocked with 3% BSA in Tris-buffered saline (50 mM Tris, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) for 4 h at room temperature. Membranes were then incubated with antibodies against phosphorylated-I κ B α (p-I κ B α ; sc-271980, Santa Cruz Biotechnology; 1:5,000), I κ B α (sc-166588, Santa Cruz Biotechnology; 1:1,000), NF- κ B p65 (sc-8008, Santa Cruz Biotechnology; 1:1,000), phosphorylated-NF- κ B p65 (p-NF- κ B p65; sc-166748, Santa Cruz Biotechnology; 1:1,000), acetyl-H3 (8173, Cell Signaling Technology, Danvers, MA; 1:1,000), histone H3 (4499, Cell Signaling Technology; 1:1,000), and GAPDH (ab8245, Abcam, Cambridge, MA; 1:5,000) overnight at 4°C, followed by incubation with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibody (Boster, Wuhan, China) for 45 min at room temperature. Specific bands were visualized with an enhanced chemiluminescence solution detection kit (ECL, Millipore, Bedford, MA). The intensity of each band was quantified using Image-Pro Plus 6.0 software (Media Cybernetics Inc., Rockville, MD).

Statistical Analysis

Each cell culture treatment was performed in triplicate, and each of the determinations were performed 3 times for each treatment. Data were analyzed using GraphPad Prism 5 (GraphPad InStat Software, San Diego, CA) and were tested for normality. Comparisons among groups were made with one-way ANOVA followed by Bonferroni correction. The results are expressed as the mean and standard error of the mean (mean $\pm$ SEM). A $P < 0.05$ was considered statistically significant.

RESULTS

Effect of SB Supplementation on Cell Viability

Compared with the control, 1 μ g/mL LPS did not change MAC-T cell viability (Figure 1). However, compared with the control, 20 μ g/mL LPS decreased MAC-T cell viability ($P = 0.0212$, Figure 1). Furthermore, compared with LPS cultures, viability was not affected by pretreatment with SB 0.1 to 5 mM plus challenge with LPS (Figure 1).

Figure 1. Effects of sodium butyrate (SB) on cell viability. Bovine mammary epithelial cells (MAC-T) were treated with 1 or 20 $\mu\text{g/mL}$ LPS for 6 h; with different concentrations of SB (0.1, 0.25, 0.5, 1, 2, or 5 mM) for 18 h; or with different concentrations of SB (0.1, 0.25, 0.5, 1, 2, or 5 mM) for 18 h, followed by a challenge with 1 $\mu\text{g/mL}$ LPS for an additional 6 h. Cell viability was determined using the Cell Counting Kit-8 assay (Dojindo, Kumamoto, Japan). Data from the control group were used to normalize the data of each treatment group. Comparisons among groups were calculated using a one-way ANOVA with subsequent Bonferroni correction. Data presented are mean $\pm$ SEM. Different lowercase letters in the bar chart indicate significant differences ($P < 0.05$).

SB Pretreatment Attenuated LPS-Induced Apoptosis

Treatment with LPS sharply increased ($P = 0.0002$) cell apoptosis compared with the control group. However, compared with LPS cultures, pretreatment with 0.25 ($P = 0.0419$), 0.5 ($P = 0.0068$), 1 ($P = 0.0058$), 2 ($P = 0.0052$), or 5 ($P = 0.0007$) mM SB decreased cell apoptosis (Figure 2).

SB Pretreatment Attenuated LPS-Induced Cytokine Production

Compared with the control cultures, LPS treatment upregulated mRNA abundance of *TNFA* ($P < 0.0001$), *IL6* ($P < 0.0001$), and *IL1B* ($P < 0.0001$; Figure 3A–C). Whereas pretreatment with 6.25, 12.5, 25, or 50 nM of the HDAC inhibitor TSA attenuated the upregulation of *TNFA* ($P = 0.0385$, $P = 0.0209$, $P = 0.0005$, or $P = 0.0003$, respectively), *IL6* ($P = 0.0421$, $P = 0.0122$, $P = 0.0002$, or $P < 0.0001$), and *IL1B* ($P = 0.0378$, $P = 0.0016$, $P < 0.0001$, or $P < 0.0001$) induced by LPS (Figure 3A–C), pretreatment with Veh did not affect mRNA abundance of *TNFA*, *IL6*, or *IL1B* (Figure 4A and B). Similarly, pretreatment with 0.1, 0.25, 0.5, 1, 2, or 5 mM SB markedly attenuated upregulation of *TNFA* ($P = 0.0391$, $P = 0.0316$, $P = 0.0007$, $P = 0.0002$, $P < 0.0001$, or $P < 0.0001$), *IL6* ($P = 0.0275$, $P = 0.0054$, $P < 0.0001$, $P < 0.0001$, $P < 0.0001$, or

$P < 0.0001$), and *IL1B* ($P = 0.0427$, $P = 0.0267$, $P = 0.0004$, $P < 0.0001$, $P < 0.0001$, or $P < 0.0001$) induced by LPS (Figure 3D–F).

SB Pretreatment Attenuated LPS-Induced Reduction of Histone H3 Acetylation

Compared with the control group, treatment with LPS reduced histone H3 acetylation ($P = 0.0083$, Figure 4A and B). However, compared with the control group, treatment with Veh increased histone H3 acetylation ($P = 0.0247$, Figure 4A and B). Compared with the LPS-treated group, pretreatment with HDAC inhibitor TSA increased histone H3 acetylation ($P < 0.0001$, Figure 4A and B), and pretreatment with Veh did not affect the histone H3 acetylation ($P = 0.6877$, Figure 4A and B). Similar to TSA, compared with the LPS-treated group, pretreatment with SB also increased histone H3 acetylation ($P = 0.0079$, Figure 4C and D).

SB Pretreatment Downregulated the LPS-Activated NF- κ B Signaling Pathway

Compared with the control group, LPS treatment increased the ratio of p-I κ B α to I κ B α ($P = 0.0050$, Figure 5A and B). In contrast, compared with the LPS-

Figure 2. Effects of sodium butyrate (SB) on apoptosis of bovine mammary epithelial (MAC-T) cells. Cells were treated with 1 $\mu\text{g/mL}$ LPS for 6 h; with different concentrations of SB (0.1, 0.25, 0.5, 1, 2, or 5 mM) for 18 h; or with different concentrations of SB (0.1, 0.25, 0.5, 1, 2, or 5 mM) for 18 h, followed by a challenge with 1 $\mu\text{g/mL}$ LPS for an additional 6 h. The percentage of apoptotic cells was determined by flow cytometry. Data from the control group were used to normalize the data of each treatment group. Comparisons among groups were calculated using a one-way ANOVA with subsequent Bonferroni correction. Data presented are mean $\pm$ SEM. Different lowercase letters in the bar chart indicate significant differences ($P < 0.05$).

Figure 3. Effects of sodium butyrate (SB) on cytokine production. Bovine mammary epithelial cells (MAC-T) were treated with 1 $\mu\text{g/mL}$ LPS for 6 h; with different concentrations of trichostatin A (TSA; 6.25, 12.5, 25, or 50 nM) for 18 h, followed by a challenge with 1 $\mu\text{g/mL}$ LPS for an additional 6 h; with amounts of solvent [0.1% (vol/vol) ethanol, vehicle control (Veh)] equal to that present in TSA cultures for 18 h, followed by a challenge with 1 $\mu\text{g/mL}$ LPS for an additional 6 h; or with different concentrations of SB (0.1, 0.25, 0.5, 1, 2, or 5 mM) for 18 h, followed by a challenge with 1 $\mu\text{g/mL}$ LPS for an additional 6 h. (A) Relative mRNA expression level of *TNFA*. (B) Relative mRNA expression level of *IL6*. (C) Relative mRNA expression level of *IL1B*. (D) Relative mRNA expression level of *TNFA*. (E) Relative mRNA expression level of *IL6*. (F) Relative mRNA expression level of *IL1B*. Data from the control group were used to normalize the data of each treatment group. Comparisons among groups were calculated using a one-way ANOVA with subsequent Bonferroni correction. Data presented are mean $\pm$ SEM. Different lowercase letters in bar charts indicate significant differences ($P < 0.05$).

treated group, pretreatment with SB reduced the ratio of p-I κ B α to I κ B α ($P = 0.0052$, Figure 5A and B). Similarly, LPS treatment also increased the ratio of p-p65 to p65 ($P = 0.0017$), whereas pretreatment with SB attenuated the upregulation of the ratio of p-p65 to p65 induced by LPS ($P = 0.0098$, Figure 5A and C). Consistent with the alterations in the ratio of p-p65 to p65, immunofluorescence staining results revealed that, compared with the control group, LPS treatment increased NF- κ B p65 translocation to the nucleus. However, compared with the LPS-treated group, NF- κ B p65 translocation to the nucleus was greatly decreased in the group treated with SB plus LPS (Figure 6).

DISCUSSION

The BMEC play an essential role in the host response by alerting the immune system to invasion of pathogens; hence, they are a major line of defense of the mammary gland (Rainard and Riollot, 2003). Dysregulation of inflammatory responses has a critical role in the progression of bovine mastitis. Cytokines

act as intercellular chemical messengers involved in the regulation of immune homeostasis and inflammatory responses against different infections (Okada et al., 1999). Among the cytokines, *TNFA*, *IL6*, and *IL1B* are considered pro-inflammatory, with various studies reporting upregulation of *TNFA*, *IL6*, and *IL1B* mRNA abundance during coliform mastitis in the transition period (Sordillo and Peel, 1992; Shuster et al., 1993). In vitro, the abundance of these cytokines in BMEC exposed to LPS also increases (Wang et al., 2016). Thus, reduction of pro-inflammatory cytokine production may be an effective strategy for treating mastitis. The downregulation of mRNA abundance of *TNFA*, *IL6*, and *IL1B* in LPS-challenged cells that were pretreated with SB underscored the positive effect of SB on the inflammatory responses of BMEC. These data agree with recent data in ruminal epithelium and bovine hepatocytes (Dai et al., 2017; Xu et al., 2018). In the absence of an inflammatory challenge, the viability of mouse mammary epithelial cells was not affected by 0.1 to 2 mM SB treatment for 18 h (Wang et al., 2017). Thus, together, the available data suggest that

Figure 4. Effects of sodium butyrate (SB) on histone H3 acetylation. Bovine mammary epithelial cells (MAC-T) were treated with 1 $\mu\text{g/mL}$ LPS for 6 h; with 0.5 mM SB for 18 h; with 25 nM trichostatin A (TSA) for 18 h; with an amount of solvent [0.1% (vol/vol) ethanol, vehicle control (Veh)] equal to that present in TSA cultures for 18 h; with 0.5 mM SB for 18 h, followed by a challenge with 1 $\mu\text{g/mL}$ LPS for an additional 6 h; with 25 nM TSA for 18 h, followed by a challenge with 1 $\mu\text{g/mL}$ LPS for an additional 6 h; or with an amount of Veh equal to that present in TSA cultures for 18 h, followed by a challenge with 1 $\mu\text{g/mL}$ LPS for an additional 6 h. (A) Western blot analysis of acetylated histone H3 and histone H3. (B) Relative protein expression levels of acetylated histone H3 and histone H3. (C) Western blot analysis of acetylated histone H3 and histone H3. (D) Relative protein expression levels of acetylated histone H3 and histone H3. Data from the control group were used to normalize the data of each treatment group. Comparisons among groups were calculated using a one-way ANOVA with subsequent Bonferroni correction. Data presented are mean $\pm$ SEM. Different lowercase letters in bar charts indicate significant differences ($P < 0.05$).

the anti-inflammatory effect of SB is not due to differences in cell numbers before LPS challenge. As such, an enhanced supply of SB might exert a preventive effect against inflammation in ruminants.

The protein NF- κ B is a nuclear transcription factor that plays a crucial role in the innate immune system. Activation of NF- κ B is involved in the inflammatory processes associated with development of mastitis (Gong et al., 2018). Upon stimulation of cells by a variety of chemical and mechanical signals that lead to phosphorylation and degradation of I κ B α , NF- κ B is phosphorylated and translocated to the nucleus, triggering transcription of inflammatory cytokines (Niederberger and Geisslinger, 2008). Thus, inhibition of NF- κ B activation has attracted attention as a therapeutic approach for intervention during immune and inflammatory events (Calzado et al., 2007).

At least in non-ruminants, the anti-inflammatory response potential of SB stems from its ability to prevent phosphorylation and degradation of I κ B α (Usami et al., 2008). Compared with cultures challenged with LPS, downregulation of the phosphorylation of I κ B α in cultures treated with SB and LPS suggested that pretreatment with SB was effective in attenuating the phosphorylation of I κ B α resulting from LPS challenge. Upon phosphorylation of I κ B α in response to LPS, a concomitant increase in phosphorylated NF- κ B p65 and production of pro-inflammatory cytokines has been reported (Lee et al., 2017), suggesting that preventing phosphorylation of I κ B α might be an important goal. The fact that cultures treated with SB and LPS decreased phosphorylated NF- κ B p65 movement into the nucleus is consistent with previous findings in bovine hepatocytes (Xu et al., 2018). Thus, the data suggest

Figure 5. Effects of sodium butyrate (SB) on NF- κ B signaling pathway. Bovine mammary epithelial cells (MAC-T) were treated with 1 μ g/mL LPS for 6 h; with 0.5 mM SB for 18 h; or with 0.5 mM SB for 18 h, followed by a challenge with 1 μ g/mL LPS for an additional 6 h. (A) Western blot analysis of p-I κ B α , I κ B α , p-p65, and p65. (B) Relative protein expression levels of p-I κ B α to I κ B α . (C) Relative protein expression levels of p-p65 to p65. Data from the control group were used to normalize the data of each treatment group. Comparisons among groups were calculated using a one-way ANOVA with subsequent Bonferroni correction. Data presented are mean $\pm$ SEM. Different lowercase letters in bar charts indicate significant differences ($P < 0.05$).

that SB pretreatment ameliorates LPS-induced activation of the NF- κ B pathway, which may help attenuate or modulate immune responses by BMEC.

The level of the inflammatory response is clearly dependent on the activation state of NF- κ B, which is tightly regulated by modification of histone acetylation status. By modifying histone acetylation, HDAC play a key role in the regulation of NF- κ B-dependent inflammation. In the clinical setting, HDAC inhibitors have already been used to treat inflammatory diseases including rheumatoid arthritis, asthma, and ischemia-reperfusion injury in humans (Granger et al., 2008; Grabiec et al., 2010; Royce and Karagiannis, 2012). Although HDAC inhibitors have not been applied to the treatment of infectious diseases, previous studies have revealed that SB is an HDAC inhibitor, which could induce histone acetylation and reduce the NF-

κ B-dependent inflammatory response (Place et al., 2005; Wang et al., 2017). The fact that Silva et al. (2018) reported that pretreatment of MAC-T cells with SB increased histone H3 acetylation underscores the possibility that, during conditions when histone acetylation is increased, the NF- κ B-dependent inflammatory response is inhibited. Along with the downregulation of phosphorylated NF- κ B p65 and reduction of inflammatory cytokine transcription, the upregulation of histone acetylation in cultures with SB pretreatment followed by LPS stimulation support the role of SB as an HDAC inhibitor, leading to dampening of the inflammatory response. Taken together, the available data confirm that SB may also be effective in the prevention of inflammatory responses induced by LPS.

Despite the acknowledged limitations of the study—for instance, protein abundance of the pro-inflammatory

cytokines was not evaluated, and SB supplementation was performed before the inflammatory insult (LPS)—the present study establishes a potential strategy for SB supplementation to prevent inflammatory responses in MAC-T cells. Based on the present data, it is expected that SB controls inflammation via regulation of mRNA abundance and phosphorylation of key proteins. Future research into BMEC and the bovine mammary gland are needed to better understand the mechanisms of action of SB against mammary inflammation.

The use of short-chain fatty acids as a feed additive continues to receive attention in the animal nutrition community. For instance, butyrate is known to enhance performance and benefit gut health in non-ruminants (Robles et al., 2013). In isolated goat mammary cells, Chang et al. (2018) reported that dietary SB supplementation reduced apoptosis induced by high-concentrate diet feeding, through the attenuating activation of NF- κ B and CASP3. Although most butyrate is converted to BHB by the ruminal epithelium, and very little butyrate makes it past the liver, the reduced concentrations of LPS and proinflammatory cytokines

reported in vivo, together with our data, indicate that an SB feed additive might be effective in preventing LPS-associated mastitis. Although attenuation of inflammation by SB may impair the sentinel function of the mammary gland and allow for infection to be established more easily, future research on the applicability of SB should determine the optimal dose that reduces inflammation without impairing the sentinel function of the mammary gland.

CONCLUSIONS

Enhanced supply of SB protects MAC-T cells against LPS-induced inflammation partly by decreasing mRNA abundance of *TNFA*, *IL6*, and *IL1B*. Downregulation of the phosphorylation of I κ B α and transport of phosphorylated NF- κ B p65 into the nucleus by SB suggest that inactivation of the NF- κ B pathway is involved in the cytoprotective effect of SB. Furthermore, the anti-inflammatory response functions of SB in MAC-T cells might be partly mediated by its upregulation of histone acetylation. Thus, control of the NF- κ B pathway and

Figure 6. Effects of sodium butyrate (SB) on the location of NF- κ B p65 protein. Bovine mammary epithelial cells (MAC-T) were treated with 1 μ g/mL LPS for 6 h; with 0.5 mM SB for 18 h; or with 0.5 mM SB for 18 h, followed by a challenge with 1 μ g/mL LPS for an additional 6 h. Afterward, immunofluorescence for NF- κ B p65 (red) was performed, and the nuclear dye 4',6-diamidino-2-phenylindole (DAPI; blue) was used. Scale bar = 25 μ m.

downstream pro-inflammatory cytokine production constitute important mechanisms for SB action against inflammation.

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