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Sodium acetate inhibits *Staphylococcus aureus* internalization into bovine mammary epithelial cells by inhibiting NF-κB activation

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ABSTRACT

Bovine mastitis is one of the most costly and prevalent disease affecting dairy cows worldwide. It was reported that *Staphylococcus aureus* could internalize into bovine mammary epithelial cells (bMEC) and induce mastitis. Some short chain fatty acids (SCFA) have shown to suppress *S. aureus* invasion into bMEC and regulate antimicrobial peptides expression. But it has not been evaluated that sodium acetate has the similar effect. The aim of this study was to investigate the effect of sodium acetate on the invasion of bovine mammary epithelial cells (bMEC) by *S. aureus*. Gentamicin protection assay showed that the invasion of *S. aureus* into bMEC was inhibited by sodium acetate in a dose-dependent manner. Sodium acetate (0.25–5 mM) did not affect *S. aureus* growth and bMEC viability. The TAP gene level was decreased, while the BNBD5 mRNA level was enhanced in sodium acetate treated bMEC. In sodium acetate treated and *S. aureus* challenged bMEC, the TAP gene expression was increased and BNBD5 gene expression was not modified at low concentrations, but decreased at high concentrations. The Nitric oxide (NO) production of bMEC after *S. aureus* stimulation was decreased by sodium acetate treatment. Furthermore, sodium acetate treatment suppressed *S. aureus*-induced NF-κB activation in bMEC in a dose manner. In conclusion, our results suggested that sodium acetate exerts an inhibitory property on *S. aureus* internalization and modulates antimicrobial peptides gene expression.

1. Introduction

Bovine mastitis is a major disease affecting dairy cattle worldwide. It can cause a decline of milk production and quality, which results in economic losses for dairy farming [1]. Mastitis leads to an increase of somatic cells in the milk [2]. This disease is characterized by inflammation of the mammary gland, which caused by a broad spectrum of bacterial and fungal pathogens [3,4]. *Staphylococcus aureus* (*S. aureus*) tends to result in chronic, subclinical mastitis and may resist antibiotic therapy [5,6]. Bacterial invasion into mammary epithelial cells is an important pathogenic mechanism for the establishment of the bovine mastitis and it could be modulated by environmental, genetic, or hormonal factors [7,8]. In previous studies, we have known that *S. aureus* responsible for bovine mastitis has the ability to internalize into bovine mammary epithelial cells (bMEC) [9,10].

Short chain fatty acids (SCFA), including acetate, propionate, butyrate and hexanoate, are derived from bacterial fermentation of undigested dietary fibre in the colon and in the forestomach of ruminants [11,12], and they are components of bovine milk [13]. In addition to their nutrimental function, the production and absorption of SCFA exert a very significant effect on the epithelial cell growth [14,15]. Besides, SCFA were found to be inhibitory towards an array of pathogenic microbes including Gram-positive bacteral *S. aureus* [16,17]. SCFA also have anti-inflammatory and immunemodulatory properties [18]. Several studies have demonstrated that sodium propionate, sodium butyrate and sodium hexanoate could reduce *S. aureus* internalization into bMEC and modulate antimicrobial peptides mRNA expression [18,19]. However, the effect of sodium acetate on *S. aureus* invasion into bMEC and antimicrobial peptide gene expression has not been evaluated.

The objective of this work was to assess the effects of sodium acetate on internalization of *S. aureus* responsible for bovine

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mastitis into bMEC and to examine its effects on the gene expression of antimicrobial peptides during the infection process.

2. Materials and methods

2.1. Strain and reagents

S. aureus subsp. *aureus* (ATCC 49525) strain isolated from the cows with subclinical mastitis was used in this study [20]. Bacteria were grown at 37 °C for 12 h in Mueller-Hinton II cation adjusted broth (MH, BD Biosciences, Sparks, MD, USA) and CFUs were adjusted by measuring optical density at 600 nm. Sodium acetate was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of reagent grade.

2.2. Primary culture of bovine mammary epithelial cells (bMEC)

bMEC were isolated from alveolar tissue of udders of locating cows as described previously [21]. Animal experiments were done in accordance with the guidelines on animal care and use established by the Jilin University Animal Care and Use Committee. The protocols were reviewed and approved by the committee. Isolated cells were placed in 60 mm dishes in growth medium (GM) composed by Dulbecco's modified Eagle's medium/nutrient mixture F12 Ham (DMEM/F12, Sigma) supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), 100 U/ml penicillin and streptomycin (100 mg/ml) and 1 mg/ml amphotericin B (Invitrogen, Carlsbad, CA, USA). Cells were grown at 37 °C with 5% CO_2.

2.3. Effect of sodium acetate on S. aureus 49525 growth and bMEC viability

To determine the effect of sodium acetate on *S. aureus* growth, 1.8×10^7 CFU/ml were cultured at 37 °C in MH broth supplemented with different concentrations of sodium acetate (0.25–5 mM) and growth was measured by optical density at 600 nm during 24 h. A CCK-8 (Cell Counting Kit-8, Dojindo Laboratories, Kumamoto, Japan) assay was used to analyze the effect of sodium acetate on bMEC viability. Briefly, bMEC were plated at a density of 1×10^4 cells/ml in 96-well plate in a 37 °C, 5% CO_2 incubator for 4 h; then the cells were treated with different concentrations of sodium acetate (0.25–5 mM) for 24 h and 48 h, followed by stimulation with 10 μl/well CCK-8 solutions. After incubated for additional 3 h in the dark, the optical density was measured at 450 nm on a microplate spectrophotometer (TECAN, Austria). bMEC cultured in medium alone and medium without cells were served as control.

2.4. Effect of sodium acetate on S. aureus 49525 internalization into bMEC

To evaluate the effect of sodium acetate on internalization of *S. aureus* into bMEC, gentamicin protection assay was carried out in our study using a procedure described previously [18,19]. Briefly, bMEC (~2×10^5) were incubated in 24-well dishes with different concentrations of sodium acetate (0.25–5 mM) dissolved in GM without antibiotics and FBS for 24 h and then were challenged with *S. aureus* (MOI 30:1 bacteria per cell), for this, bMEC were cultured with 20 μl of bacterial suspensions at a density of 6×10^6 CFU/ml for 2 h in 5% CO_2 at 37 °C. Later on, bMEC were washed three times with PBS (pH 7.4) and incubated in GM without FBS supplemented with 50 μg/ml gentamicin for 1 h in 5% CO_2 at 37 °C to kill extracellular bacteria. Subsequently, bMEC were washed by PBS for three times again; followed by detached with Trypsin (0.25%, supplemented with 0.1% EDTA-2Na, Sigma) and lysed with 400 μl of sterile distilled water. The bMEC lysates were collected and diluted 200-fold, plated on MH agar in triplicates overnight at 37 °C. Total CFU was determined by the standard colony counting technique. Data was presented as the percentage of internalization in relation to bMEC cultured with the vehicle.

2.5. Total RNA isolation and quantitative real-time PCR

bMEC were seeded in 6-well plates with various concentrations of sodium acetate for 24 h followed by infecting with or without *S. aureus* for 2 h. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA samples were treated with DNase I (MBI Fermentas, Lithuania) to prevent genomic DNA contamination. The RNA was reverse-transcribed into cDNA using the Revert Aid First Strand cDNA Synthesis Kit (MBI Fermentas, Lithuania). The quantification of relative mRNA concentrations was detected by quantitative real-time polymerase chain reaction (qRT-PCR) using a 7500 Fast Real-Time PCR System (Applied Biosystems, Carlsbad, California, USA). The reactions were incubated at 50 °C for 2 min and then incubated at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min, using the FastStart Universal SYBR Green Master (Roche Applied Science, Mannheim, Germany) according to the manufacturer's instructions. Specific primers used for qRT-PCR were listed in Table 1. Each reaction run three times, the threshold cycle (C_t) values (Rotor-Gene software, Corbett Research, version 1.7) and PCR efficiently averaged. $2^{-\triangle\triangle t}$ values were chosen to reflect the mRNA expression, and GAPDH was used as an internal control [22].

2.6. Nitrite concentration assays

In order to analyze the Nitric oxide (NO) production of bMEC, accumulation of nitrite (NO_2^-) in the culture medium was measured by using Greiss reaction. Briefly, bMEC was treated with various concentrations of sodium acetate in the presence or absence of *S. aureus,* then culture supernatant was collected and mixed with the same volume of Greiss reagent I (10% sulfanilamide, 40% phosphoric acid, Sigma) and Greiss reagent II (1% N -(1-naphthyl) -ethylenediamine -dihydrochloride, Sigma) to detect nitrite. The absorbance at 570 nm was determined using a microplate spectrophotometer (TECAN). Nitrite concentration was calculated with reference to a standard curve of sodium nitrite.

2.7. Western blot analysis

bMEC were seeded in 6-well plates and incubated for 24 h. Then the cells were treated with various concentrations of sodium acetate for 24 h before infecting with *S. aureus* at a multiplicity of infection of 30 (MOI 30:1 bacteria per cell). Then, 20 μl of bacterial suspensions with 6×10^6 *staphylococci* were added in culture medium for 45 min, the cells were collected and washed twice with cold PBS. Proteins from bMEC were extracted using Nuclear and Cytoplasmic Protein Extraction Kit (Thermo) according to the manufacturer's protocol. Protein concentration was determined through BCA method. The proteins (30 μg) were separated by SDS-PAGE using Tris-HCl Precast Gels and then transferred onto the PVDF membrane. The resulting membrane was blocked with phosphate buffer solution containing 0.05% Tween-20 (PBS-T), supplemented with 3% skim milk at room temperature for 2 h on a rotary shaker, and followed by PBS-T washing. The specific primary antibody diluted in PBS-T containing skim milk, was incubated with the membrane at 4 °C overnight. Subsequently, the membrane was washed with PBS-T followed by incubation with the secondary antibody conjugated with horseradish peroxidase at room temperature for 1 h. Blots were again washed with PBS-T and then developed with the ECL Plus Western Blotting Detection System