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Short chain fatty acids (propionic and hexanoic) decrease *Staphylococcus aureus* internalization into bovine mammary epithelial cells and modulate antimicrobial peptide expression

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ABSTRACT

Short chain fatty acids (SCFAs) are critical nutrients for ruminants and are mainly obtained from bacterial fermentation of carbohydrates. In addition to their nutrimental function, SCFAs have antimicrobial and anti-inflammatory properties, as well as immunomodulatory roles. It has been reported that sodium butyrate reduces *Staphylococcus aureus* internalization into bovine mammary epithelial cells (bMEC) and modulates antimicrobial peptide mRNA expression. Nevertheless, it has not been evaluated if sodium propionate (NaP) and sodium hexanoate (NaH) have similar actions. Since they are present in milk, the aim of this study was to determinate the effect of both SCFAs on *S. aureus* internalization into bMEC and to evaluate their effects on modulation of innate immunity elements. Our data showed that both SCFAs (0.25–5 mM) did not affect *S. aureus* growth and bMEC viability. By gentamicin protection assay (MOI 30:1) we showed that NaP and NaH reduced bacterial internalization into bMEC, which ranged 27–55% and 39–65%, respectively, in relation to non treated controls. Also, both SCFAs up-regulate tracheal antimicrobial peptide (TAP) mRNA expression; however, bovine neutrophil β-defensin 5 (BNBD5) mRNA expression was not modified or was down-regulated. In addition, TAP and BNBD5 expression was up-regulated by *S. aureus*. Finally, the decrease in bacterial internalization under SCFA treatments is not related to nitric oxide production. In conclusion, NaP and NaH decrease *S. aureus* internalization into bMEC and modulate TAP gene expression, which may be related to the reduction in bacterial internalization.

1. Introduction

Bovine mastitis is the most important infectious disease of dairy cattle that results in economic losses and decreased animal health (Ruegg, 2003). The predominantly contagious pathogen responsible for mastitis in dairy cows is *Staphylococcus aureus* that has the ability to internalize into epithelial and endothelial cells (Almeida et al., 1996, 2007; Hensen et al., 2000; Kerro-Dego et al., 2002; Strandberg et al., 2005).

Short chain fatty acids (SCFAs) are well known as a major energy source for ruminants (~70%); also are important components of bovine milk (Bergman, 1990; Parodi, 2004). However, other roles for SCFAs have been established in cell differentiation, proliferation, motility, apoptosis and immune regulation (Li and Elsasser, 2005; Al-Lahham et al., 2010; Meijer et al., 2010).

A few studies have shown the role of SCFAs on the internalization of pathogens into epithelial cells. Propionic and butyric acids decrease *Salmonella enteritidis* internalization into avian intestinal epithelial cells (Van Immerseel et al., 2003). Pretreatment of *Salmonella* with SCFAs decreases its internalization into chicken cecal epithelial cells and into the intestinal epithelial cell line

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T84 (Van Immerseel et al., 2004a,b). On the other hand, butyrate protects Caco-2 cells from *Campylobacter jejuni* invasion (Van Deun et al., 2008). Also, we have demonstrated that butyrate reduces *S. aureus* internalization into bovine mammary epithelial cells (bMEC) and up-regulates the TAP, β-defensin and inducible nitric oxide synthase (iNOS) mRNAs expression, as well as nitric oxide production (Ochoa-Zarzosa et al., 2009). However, the effects of other SCFAs, as sodium propionate (NaP) and sodium hexanoate (NaH) on *S. aureus* internalization into bMEC and antimicrobial peptide gene expression have not been evaluated.

The objective of this work was to assess the role of NaP and NaH on *S. aureus* internalization responsible of mastitis into bMEC. In addition, we evaluated the antimicrobial peptide gene expression and nitric oxide production during this process.

2. Materials and methods

2.1. Strain and reagents

S. aureus subsp. *aureus* (ATCC 27543) strain isolated from a case of bovine clinical mastitis was used in this study. This strain has recognized capacity to internalize into bovine mammary epithelial cells (Gutiérrez-Barroso et al., 2008). Bacteria were grown at 37 °C overnight in Luria-Bertani broth (LB, Bioxon, México). NaP and NaH were acquired from Sigma (St. Louis, MO, USA).

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

The isolation of bMEC was performed from alveolar tissue of lactating cows udders as described (Anaya-López et al., 2006). Cells from passages 2nd to 8th were cultured in Petri dishes (Corning-Costar, New York, USA) in growth medium (GM) composed by DMEM medium/nutrient mixture F-12 Ham (DMEM/F-12K, Sigma) supplemented with 10% fetal calf serum (Equitech-Bio Inc, Kerrville, TX, USA), 10 μg/ml insulin (Sigma), 5 μg/ml hydrocortisone (Sigma), 100 U/ml penicillin and streptomycin (100 μg/ml) and 1 μg/ml amphotericin B (Invitrogen, Carlsbad, CA, USA). Cells were grown in 5% CO_2 atmosphere at 37 °C.

2.3. Effect of SCFAs on S. aureus 27543 growth and bMEC viability

To analyze SCFAs effect on *S. aureus* growth, 9×10^7 CFU/ml were cultured at 37 °C in LB broth supplemented with different concentrations of NaP or NaH (0.25–5 mM) and growth was monitored turbidimetrically (600 nm) after 24 h. To determine the effect of SCFAs on bMEC viability, 5×10^3 cells were incubated with SCFAs (0.25–5 mM) during 24–48 h at 37 °C in a 96-well flat-bottom plate. Then, 10 μl of 5 mg/ml of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT, Sigma) solution in phosphate buffer saline (PBS) was added to each well and incubated during 4 h at 37 °C. Finally, 100 μl of acid isopropanol (95% isopropanol and 5% of 1 N HCl) was added to dissolve formazan crystals. Optical density was measured with a microplate spectrophotometer (DAS) at 595 nm and compared with untreated controls.

2.4. Effect of SCFAs on internalization of S. aureus 27543 into bMEC

In this study we used bMEC polarized monolayers that were created on plates coated with 6–10 μg/cm^2 rat-tail type I collagen (Sigma). Internalization assays (gentamicin protection assay) were carried out as described (Ochoa-Zarzosa et al., 2009). Briefly, prior to assays bMEC were incubated with different SCFA concentrations (0.25–5 mM) for 24 h. bMEC monolayers ($\sim 2 \times 10^5$ cells/well) were challenged with *S. aureus* (30:1 bacteria per cell), for this, bMEC were inoculated with 65 μl of bacterial suspensions to 9.2×10^7 CFU/ml and incubated for 2 h in 5% CO_2 at 37 °C. After, bMEC monolayers were washed three times with PBS (pH 7.4) and incubated in GM without serum, supplemented with 50 μg/ml gentamicin for 1 h at 37 °C to eliminate extracellular bacteria. Then, bMEC monolayers were detached with trypsin-EDTA (Sigma) and lysed with 250 μl of sterile distilled water. bMEC lysates were diluted 100-fold, plated on LB agar for triplicate and incubated overnight at 37 °C. The number of total CFU was determined by the standard colony counting technique. Data are presented as the percentage of internalization in relation to untreated bMEC.

2.5. RNA isolation and antimicrobial peptide gene expression analysis

bMEC total RNA (5 μg) was extracted from all conditions evaluated with Trizol reagent (Invitrogen) according to manufacturer's instructions, and then used to synthesize cDNA. Genomic DNA contamination was removed from RNA samples with DNase I treatment (Invitrogen). Reverse transcription (RT) reaction was performed in 20 μl containing 25 μg/ml Oligo d(T) (Invitrogen) and 500 nM dNTPs (Invitrogen). The reaction was incubated at 65 °C for 5 min, and immediately transferred to ice. Then, 1× first strand buffer (Invitrogen), 10 mM dithiothreitol and 2 U/μl RNAse inhibitor (Invitrogen) were added to the reaction mixture and incubated at 37 °C for 2 min. Finally, 10 U/μl M-MLV reverse transcriptase (Invitrogen) was added and the mixture was incubated again at 37 °C for 50 min, followed by 70 °C for 15 min.

The relative quantification of gene expression (qPCR) was performed using the comparative Ct method (ΔΔCt) in a StepOne Plus Real-Time PCR System (Applied Biosystems) according to manufacturer's instructions. The reactions were carried out with a SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA, USA). Specific primers were used to amplify genes encoding antimicrobial peptides BNBD5 and TAP (Table 1). GAPDH was used as an internal control (endogenous gene).

2.6. Determination of nitrite concentration

Nitric oxide (NO) secreted by bMEC to culture medium was evaluated by measuring the nitrite concentration