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Cholecalciferol (vitamin D) differentially regulates antimicrobial peptide expression in bovine mammary epithelial cells: Implications during *Staphylococcus aureus* internalization

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ABSTRACT

Vitamin D has immunomodulatory functions regulating the expression of host defense genes. The aim of this study was to determine the effect of cholecalciferol (vitamin D3) on *S. aureus* internalization into bovine mammary epithelial cells (bMEC) and antimicrobial peptide (AP) mRNA expression. Cholecalciferol (1–200 nM) did not affect *S. aureus* growth and bMEC viability; but it reduced bacterial internalization into bMEC (15–74%). Also, bMEC showed a basal expression of all AP genes evaluated, which were induced by *S. aureus*. Cholecalciferol alone or together with bacteria diminished tracheal antimicrobial peptide (TAP) and bovine neutrophil β-defensin (BNBD) 5 mRNA expression; while alone induced the expression of lingual antimicrobial peptide (LAP), bovine β-defensin 1 (DEFB1) and bovine psoriasin (S100A7), which was inhibited in the presence of *S. aureus*. This compound (50 nM) increased BNBD10 mRNA expression coinciding with the greatest reduction in *S. aureus* internalization. Genes of vitamin D pathway (25-hydroxylase and 1 α-hydroxylase) show basal expression, which was induced by cholecalciferol or bacteria. *S. aureus* induced vitamin D receptor (VDR) mRNA expression, but not in the presence of cholecalciferol. In conclusion, cholecalciferol can reduce *S. aureus* internalization and differentially regulates AP expression in bMEC. Thus, vitamin D could be an effective innate immunity modulator in mammary gland, which leads to a better defense against bacterial infection.

1. Introduction

Vitamin D has a well-known role as regulator of calcium homeostasis and is critical for bone mineralization (Macdonald et al., 2010). In addition, vitamin D has been associated with various regulatory effects on the immune system (Miller and Gallo, 2010). The biologically active metabolite 1α,25-dihydroxyvitamin D3 (1,25D3) is produced by sequential hydroxylations of the precursor cholecalciferol (vitamin D3) in the liver and kidney by the enzymes 25-hydroxylase (CYP27A1) and 1 α-hydroxylase (CYP27B1), respectively (Krishnan and Feldman, 2011); although these enzymes can be expressed in other tissues (Cross, 2007). Also, the extrarenal expression of 1 α-hydroxylase has been reported in cattle (Nelson et al., 2010a). 1,25D3 binds to nuclear VDR and acts as a transcription factor for genes that contain vitamin D response elements (VDRE) (Aranow, 2011). In humans, one of the most prominent gene up-regulated by 1,25D3 is the AP cathelicidin (Gombart et al., 2005). However, in bovine

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monocytes 1,25D3 does not stimulate the expression of this gene family (Nelson et al., 2010a), and it is unknown whether vitamin D can regulate the expression of other AP in bovine cells during an infectious process.

Staphylococcus aureus responsible of bovine mastitis has the ability to internalize into bMEC (Almeida et al., 1996; Kerro-Dego et al., 2002). In previous studies, we have shown that the short chain fatty acids propionic, butyric and hexanoic, some of them components of bovine milk, inhibit *S. aureus* internalization into bMEC and regulate the expression of innate immunity response genes (Ochoa-Zarzosa et al., 2009; Alva-Murillo et al., 2012). The role of other milk components, such as vitamin D, on this event remains unknown. The main purpose of this work was to evaluate the effect of cholecalciferol on *S. aureus* internalization into a primary culture of bMEC and on the expression of diverse AP and vitamin D pathway genes.

2. Materials and methods

2.1. Strain and reagents

S. aureus subsp. *aureus* (ATCC 27543) strain was used in this study. This strain can be internalized into bMEC and was obtained from a case of bovine clinical mastitis (Gutiérrez-Barroso et al., 2008). Inoculum was prepared from bacteria that were grown at 37 °C overnight in Luria-Bertani broth (LB, Bioxon, México). Cholecalciferol (vitamin D3) was acquired from Sigma (St. Louis, MO, USA) and the working solutions were dissolved in ethanol. Bovine plasma concentrations of 25-hydroxyvitamin D3 (25D3) are 20–50 ng/ml (50–125 nM) (McDermott et al., 1985). Based on these data we established a range of concentrations of 1–200 nM. In all experiments ethanol (vehicle) was used as a control.

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

bMEC isolation 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 cell culture dishes (6, 24 or 96 wells, 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. Effects of cholecalciferol on S. aureus 27543 growth and bMEC viability

To analyze the effect of cholecalciferol on *S. aureus* growth, 9×10^7 CFU/ml were cultured at 37 °C in LB broth supplemented with different concentrations of this molecule (1, 10, 50, 100 and 200 nM) and growth was monitored turbidimetrically (600 nm) during 48 h. To determine the effect of cholecalciferol on bMEC viability, 5×10^3 cells were incubated with this molecule (1–200 nM) for 24 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, Rome, Italy) at 595 nm and was compared with the control (cells treated with vehicle).

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

The effect of cholecalciferol on *S. aureus* internalization into bMEC was evaluated by gentamicin protection assay as described (Ochoa-Zarzosa et al., 2009). Briefly, we used bMEC polarized monolayers ($\sim 2 \times 10^5$ cells cultured in 24 well dishes with 6–10 μg/cm^2 rat-tail type I collagen, Sigma) which were incubated with different cholecalciferol concentrations (1–200 nM) for 24 h and then were infected with *S. aureus* (MOI 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 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. Finally, 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 in triplicates and incubated overnight at 37 °C. CFU total was determined by the standard colony counting technique. Data are presented as the percentage of internalization in relation to control (bMEC treated with vehicle).

2.5. RNA isolation and antimicrobial peptide gene expression analysis

RNA extraction from bMEC cultured in 6 well dishes was carried out using Trizol reagent (Invitrogen) according to manufacturer's instructions. Genomic DNA contamination was removed from RNA samples with DNase I treatment (Invitrogen). RNA was reverse transcribed to cDNA in a 20 μl reaction 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 was transferred to ice. Then, 1X First Strand Buffer (Invitrogen), 10 mM dithiothreitol (Sigma) and 2 U/μl RNAse inhibitor (RNAseOUT, 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.

Real-time PCR was performed using a StepOne Plus Real-Time PCR System (Applied Biosystems, Carlsbad, California, USA). The reactions were incubated at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Each reaction contained 5 μl SYBR Green PCR Master Mix (Applied Biosystems), 0.9 μl each of 10 mM forward and reverse primers, and 250 ng of cDNA. Primer pairs were acquired from Invitrogen (Table 1) and the