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Prolactin stimulates the internalization of *Staphylococcus aureus* and modulates the expression of inflammatory response genes in bovine mammary epithelial cells

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Abstract

The incidence of mastitis in dairy cattle is highest at the drying off period and parturition, which are characterized by high levels of the lactogenic hormone prolactin (PRL). One of the most frequently isolated contagious pathogens causing mastitis is *Staphylococcus aureus*. However, the role of PRL on *S. aureus* infection in mammary epithelium has not been studied. In this work we evaluated the effect of bovine PRL (bPRL) on *S. aureus* internalization in a primary culture of bovine mammary epithelial cells (bMEC) and on the expression of cytokine and innate immune response genes. Our data show that 5 ng/mL bPRL enhances ~3-fold the internalization of *S. aureus* (ATCC 27543) into bMEC. By RT-PCR analysis, we showed that bPRL is able to up-regulate the expression of tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) and inducible nitric oxide synthase (iNOS) mRNAs. However, bPRL together with *S. aureus* did not modify the expression of TNF-α and iNOS mRNAs, while it down-regulated the expression of β-defensin and IL-1β mRNAs, as well as nitric oxide production, suggesting that infection and bPRL together can inhibit elements of the host immune response. To our knowledge, this is the first report that shows a role of bPRL during the internalization of *S. aureus* into bMEC.
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Keywords: Prolactin; Mastitis; Mammary epithelium; *Staphylococcus aureus*; Cytokines; iNOS

1. Introduction

Bovine mastitis is the most important infectious disease of dairy cattle, affecting both the quality and quantity of milk produced in the world. This disease is characterized by an inflammatory response of the mammary tissue caused by bacterial and fungal infections. The highest incidence of udder infection and the establishment of mastitis occur during the drying off period and around calving (Burvenich et al., 1999; Burton and Erskine, 2003). These periods are characterized by important physiological changes related to milk production and metabolism. However, the functions of lactogenic hormones such as prolactin (PRL) during the udder infection and the establishment of mastitis remain unclear. Recently, it has been

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reported that during bovine experimentally induced chronic mastitis plasma PRL concentrations did not differ between healthy and infected cows (Hockett et al., 2000; Boutet et al., 2007). Through bovine lactation and calving, PRL stimulates metabolic functions of epithelial cells, maintains the concentrations of mRNA for synthesis of milk proteins and influences the milk production together with growth hormone (GH) (Neville et al., 2002; Svennersten-Sjaunja and Olsson, 2005). In addition to the metabolic and lactogenic role of PRL, this hormone has been considered as a cytokine able to modulate the inflammatory response of the mammary epithelium (Brand et al., 2004; Boutet et al., 2007).

The most common infective agents causing mastitis in dairy cattle are pathogenic coliform environmental bacteria and *Staphylococcus* species acquired by contagious transfer (Watts, 1988; Yancey, 1999). *Staphylococcus aureus* is one of the most frequently isolated contagious pathogen causing mastitis, which may result in chronic infections characterized by the colonization of mammary tissue and the survival of the bacteria within epithelial cells (Kerro-Dego et al., 2002). The invasive ability of bacteria can be evaluated *in vitro* by measuring their capacity to internalize in cultures of bovine mammary epithelial cells. Secretory epithelial cells respond to bacterial intrusion and play a major role in the initiation of inflammation (Burton and Erskine, 2003; Alluwaimi, 2004). The *in vitro* challenge of bovine mammary epithelial cells (bMEC) with lipoteichoic acid, a gram-positive bacterial cell wall component, induces an up-regulation of the gene expression for cytokines IL-1β, TNF-α and IL-8 and for the antimicrobial peptide β-defensin (Strandberg et al., 2005). Additionally, under inflammatory conditions, bMEC are able to produce nitric oxide (NO) (Boulanger et al., 2001), which is formed by the inducible nitric oxide synthase (iNOS) in mammary gland (Onona and Inano, 1998). In immortalized bovine mammary epithelial cells (MAC-T), PRL enhances the expression of several cytokines and modulates the expression of iNOS in fibroblasts (Corbacho et al., 2003; Boutet et al., 2007). However, there is no evidence that PRL regulates the internalization of *S. aureus* into mammary epithelium.

The main purpose of this work was to evaluate the effect of bovine PRL (bPRL) on the internalization of *S. aureus* into a primary culture of bMEC. Additionally, we analyzed the effect of bPRL on the expression of TNF-α, IL-1β, β-defensin, and iNOS mRNAs, as well as on NO production in bMEC infected with *S. aureus*.

2. Materials and methods

2.1. Culture of primary bovine mammary epithelial cells

The isolation of bMEC was performed from alveolar tissue of udders of lactating cows as described previously (Anaya-López et al., 2006). Cells from passages second to eighth were cultured in Petri dishes (Corning–Costar) in growth medium (GM) composed by Dulbecco's modified Eagles's medium/nutrient mixture F-12 Ham (DMEM/F-12, Sigma) supplemented with 10% fetal calf serum (FCS) (Equitech-Bio), 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).

2.2. Invasion assays

The American Type Culture Collection (ATCC) *S. aureus* subsp. *aureus* 27543 (kindly donated by V.M. Baizabal-Aguirre, CMEB-FMVZ-UMSNH, México) strain isolated from a case of clinical mastitis was used in this study. Bovine prolactin (bPRL) was generously provided by C. Clapp (Instituto de Neurobiología, UNAM, México). Polarized monolayers of bMEC were created on plates coated with 6–10 μg/cm^2 rat-tail type I collagen (Sigma). Prior to invasion assays bMEC were incubated with different concentrations of bPRL (1–50 ng/mL) dissolved in GM without antibiotics and serum for 24 h. Then, confluent bMEC monolayers in 24-well plates (Corning–Costar) containing $\sim 2 \times 10^5$ cells/well were infected with a multiplicity of infection (MOI) of 30:1 bacteria per cell. For this, bMEC monolayers were washed three times with phosphate buffer saline (PBS, pH 7.4) and inoculated with 500 μL of bacterial suspensions to a density of 6×10^6 CFU/mL in Luria Bertani (LB) broth from overnight cultures, and incubated for 2 h in 5% CO_2 at 37 °C. After infection, bMEC monolayers were washed three times with PBS and incubated in GM without serum supplemented with 50 μg/mL gentamicin (invasion assay medium) for 2 h at 37 °C to eliminate extracellular bacteria. Subsequently, bMEC monolayers were washed three times with PBS, 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. Gentamicin-killed bacteria were used as negative control and were prepared using 50 μg/mL gentamicin (Sigma) for 2 h at 37 °C previous to

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