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Non-classical effects of prolactin on the innate immune response of bovine mammary epithelial cells: Implications during *Staphylococcus aureus* internalization

Ivan Medina-Estrada, Nayeli Alva-Murillo, Joel E. López-Meza, Alejandra Ochoa-Zarzosa*

Centro Multidisciplinario de Estudios en Biotecnología, Facultad de Medicina Veterinaria y Zootecnia, Universidad Michoacana de San Nicolás de Hidalgo, Km 9.5 Carretera Morelia-Zinapécuaro, Posta Veterinaria, C.P. 58893, Morelia, Michoacán, Mexico

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ABSTRACT

Staphylococcus aureus has the ability to invade mammary epithelial cells (bMECs) causing mastitis. This event depends primarily on the α5β1 integrin in the host cell. In addition, bMECs are a target for the hormone prolactin (PRL), which can regulate β1 integrin-dependent actions related to differentiation and lactation. Previously, we demonstrated that bovine PRL (bPRL, 5 ng/ml) stimulates *S. aureus* internalization into bMECs. TLR2 is important during *S. aureus* infections, but its activation by PRL has not yet been established. The objective of this study was to determine the role of α5β1 integrin and TLR2 during *S. aureus* internalization into bMECs stimulated with bPRL. We demonstrated that the prolactin-stimulated internalization of *S. aureus* decreases in response to the blockage of α5β1 integrin (~80%) and TLR2 (~80%). bPRL increases the membrane abundance (MA) of α5β1 integrin (~20%) and induces TLR2 MA (~2-fold). *S. aureus* reduces the α5β1 integrin MA in bMECs treated with bPRL (~75%) but induces TLR2 MA in bMECs (~3-fold). Bacteria and bPRL did not modify TLR2 MA compared with the hormone alone. *S. aureus* induces the activation of the transcription factor AP-1, which was inhibited in bMECs treated with bPRL and infected. In general, bPRL induces both pro- and anti-inflammatory responses in bMECs, which are abated in response to bacterial challenge. Interestingly, the canonical Stat-5 transcription factor was not activated in the challenged bMECs and/or treated with bPRL. Taken together, these results support novel functions of prolactin as a modulator of the innate immune response that do not involve the classical prolactin pathway.

1. Introduction

Prolactin (PRL) is a hormone involved in reproduction and lactation that essentially stimulates the differentiation and metabolic functions of mammary epithelial cells (MECs) [1]. In bovines, PRL maintains the concentrations of the mRNAs encoding milk proteins and regulates milk production [2]. The bovine mammary gland undergoes mastitis, which is characterized by an inflammatory response of the udder. The main gram-positive bacterium causing subclinical mastitis is *Staphylococcus aureus*, which can persist intracellularly in this tissue [3]. The host cell invasion in non-professional phagocytic cells (i.e., MECs) by *S. aureus* depends on the presence of fibronectin-binding proteins (FnBPs) on the bacterial surface, as well as the interaction of fibronectin and the host-cell α5β1 integrin through a zipper-type mechanism, where integrin is taken up together with its ligands. This mechanism of endocytosis is employed during the internalization process of *S. aureus* by different epithelial cells [4,5] and has also been described in a mouse model of mastitis [6].

Integrins are host-cell surface receptors that facilitate the interaction of the host cytoskeleton with proteins of the extracellular matrix (ECM) to regulate host cell differentiation, proliferation, migration, and/or adhesion [7]. These proteins consist of cell membrane-associated glycoproteins composed of one α subunit and one β subunit that form a heterodimeric complex [8]. The β1 integrin facilitates the endocytosis of several bacterial species by different types of epithelial cells [7,9]. This integrin family is very important in MECs because these proteins have been implicated in the proliferation and differentiation of MECs. Primary MECs that lack the β1 integrin have several defects, are unable to differentiate into milk-producing cells, and form acinar-like structures with clear lumens [10]. In addition, mice lacking the β1 integrin show insufficient alveolar development during pregnancy and decreased

* Corresponding author.
E-mail addresses: ochoaz@umich.mx, aleocho@hotmail.com (A. Ochoa-Zarzosa).

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lactation [11]. Because MECs respond to hormonal stimuli to proliferate and differentiate during pregnancy and lactation, there is a very important crosstalk between the lactogenic trigger, such as prolactin, and the events controlled by β1 integrin [12]. The classical PRL signaling pathway in the lactating mammary gland involves the dimerization of the PRL receptor (PRLR) and the activation of the associated tyrosine kinase Janus kinase 2 (JAK2), which eventually activates members of the protein family of signal transducer and activation of transcription (Stats) that regulate the transcriptional response to PRL related to the expression of differentiation-related genes and genes encoding milk proteins. This canonical PRL signaling is dependent on β1 integrins [12].

In a previous study, we showed that bovine PRL (bPRL) stimulates *S. aureus* internalization into bovine mammary epithelial cells (bMECs). In addition, bMECs treated with bPRL and infected with *S. aureus* show a decreased innate immune response because the bacteria inhibit the activation of nuclear factor kappa B (NF-κB) stimulated by bPRL (alone). These data described novel non-classical actions of PRL [13]. However, the relationship between bPRL and α5β1 integrin during *S. aureus* internalization into bMECs has not been explored. In addition, diverse reports indicate that the drying off and calving periods exhibit the highest incidence of udder infection due to the establishment of mastitis; these periods are characterized by the presence of high levels of PRL, and our data and those of others suggest that hormonal changes may decrease the innate immune response of calves [14,15]. Because PRL also regulates transcription factors related to the innate immune response, such as NF-κB, it is interesting to analyze the key elements of this signaling pathway in bMECs infected with *S. aureus*, i.e., Toll-like receptor 2 (TLR2). TLR2 belongs to the family of host receptors that recognize pathogen-associated molecular patterns (PAMPs). In particular, TLR2 is activated by the lipid anchor of lipoteichoic acid, lipopeptides and lipoproteins which are components of the cell membrane of gram-positive bacteria [16]. In addition, it has been shown that *S. aureus* strains activate TLR2 and its gene expression in bMECs [17]. Even though PRL may regulate many immune functions, neither a clear relationship with TLR2 nor a crosstalk between the PRLR and TLR2 pathways have been established. In contrast, the interaction of β1 integrin and TLR2 receptors has been demonstrated during infections [18]. In this sense, gram-positive bacteria can be internalized more efficiently into monocytes and macrophages because these bacteria activate integrin through the action of TLR2 [19]. In addition, the treatment with cytokines members of the tumor necrosis factor (TNF) family induces an upregulation and redistribution of β1 integrin in epithelial cells [20], and the blocking of α5β1 integrin decreases TNF-α production in response to heat-killed *S. aureus* [21].

Due to the relationship between integrins and TLRs during infection and the important crosstalk between the PRL and α5β1 integrin pathways during the proliferation and differentiation of MECs, we investigated whether bPRL stimulates the expression of integrin α5β1 in bMECs and whether this induction is related to the stimulation of the internalization of *S. aureus* by the hormone. In addition, because PRL may compromise the innate immune response of bMECs, we also explored whether this effect in combination with the stimulation of bacterial endocytosis can be achieved through the regulation of the expression and activation of TLR2 as well as other signal pathways related to the innate immune response of bMECs.

2. Materials and methods

2.1. Reagents

Native purified bovine prolactin (bPRL) (lot AFP7170E) was provided by A. F. Parlow (NHPP, NIDDK, Torrance, CA, USA), dissolved in water, and sterilized by filtration.

2.2. Antibodies

The blocking antibody anti-α5β1 integrin (MAB2514) was purchased from Millipore. The blocking anti-TLR2 (TL2.1) and anti-PRLR (U5) antibodies were obtained from Abcam. TRITC and FITC-conjugated secondary antibodies against mouse and rat IgGs were purchased from Invitrogen and Thermo Scientific, respectively.

2.3. Staphylococcus aureus strain

To perform the invasion assays, the American Type Culture Collection (ATCC) *S. aureus* subsp. *aureus* 27,543 strain was used. This strain was isolated from a case of clinical mastitis and has the capacity to invade bovine mammary epithelial cells [20]. The bacteria were grown at 37 °C overnight in Luria–Bertani broth (LB, Bioxon), and the CFUs were adjusted by measuring the optical density at 600 nm (O. D. $0.2 = 9.2 \times 10^7$ CFU/ml).

2.4. Culture of primary bovine mammary epithelial cells

bMECs were isolated from the alveolar tissue of the udders of healthy lactating cows as described [22]. Cells from passages 2 to 8 were used in all of the experiments. Cells were cultured in growth medium (GM) composed by DMEM medium/nutrient mixture F12 Ham (DMEM/F12K, Sigma) supplemented with 10% fetal calf serum (Gibco), 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). Cells were grown in 5% CO_2 atmosphere at 37 °C.

2.5. Invasion assays

For the invasion assays, we used bMEC polarized monolayers (~2×10^5 cells cultured in 24 well dishes) which were incubated with bPRL (5 ng/ml) in DMEM/F12K (Sigma) without antibiotics and serum for 24 h and then were infected with *S. aureus* (MOI 30:1 bacteria per cell). For this, bMECs 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. Then, bMECs were washed three times with PBS (pH 7.4) and incubated in GM without serum and penicillin and streptomycin, but supplemented with 50 μg/ml gentamicin for 1 h at 37 °C to eliminate extracellular bacteria. Finally, bMEC monolayers were detached with trypsin (0.05%)-EDTA (0.02%) (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. The number of CFU was determined by the standard colony counting technique. Simultaneously, the number of bMECs cultured in each well was calculated for each invasion assay using a haemocytometer. The data are presented as the ratio of CFU recovered per bMEC. For the RNA and nuclear protein extraction, bMECs were processed according to the protocols described below. For the ELISA determinations, the media of the infection assays were collected (see below).

For the invasion assays in the presence of the blocking antibodies, one hour prior to the addition of the bacteria to the bMECs, the blocking antibodies anti-α5β1 integrin (concentrations and incubation times employed according to Kapetanovic et al. [21]) or anti-TLR2 (according to the manufacturer's instructions) were added separately to triplicate wells. Rat IgGs (purified from normal rat serum with protein A-sepharose beads (Sigma)) or mouse IgGs (purified from normal mouse serum purchased from Pierce) were