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Short communication: Differential loss of bovine mammary epithelial barrier integrity in response to lipopolysaccharide and lipoteichoic acid

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ABSTRACT

In the mammary gland, the blood–milk barrier prevents an uncontrolled intermixture of blood and milk constituents and hence maintains the osmotic gradient to draw water into the mammary secretion. During mastitis, the permeability of the blood–milk barrier is increased, which is reflected by the transfer of blood constituents into milk and vice versa. In this study, we aimed to investigate changes in the barrier function of mammary epithelial cells in vitro as induced by cell wall components of different pathogens. Primary bovine mammary epithelial cells from 3 different cows were grown separately on Transwell (Corning Inc., Corning, NY) inserts. The formation of tight junctions between adjacent epithelial cells was shown by transmission electron microscopy and by immunofluorescence staining of the tight junction protein zona occludens-1. The integrity of the epithelial barrier was assayed by means of transepithelial electrical resistance, as well as by diffusion of the fluorophore Lucifer yellow across the cell layer. The release of lactate dehydrogenase (LDH) was used as an indicator for cytotoxic effects. In response to a 24-h challenge with bacterial endotoxin, barrier integrity was reduced after 3 or 7 h, respectively, in response to 0.5 mg/mL lipopolysaccharide (LPS) from *Escherichia coli* or 20 mg/mL lipoteichoic acid (LTA) from *Staphylococcus aureus*. No paracellular leakage was observed in response to 0.2 mg/mL LPS or 2 mg/mL LTA. Although LPS and LTA affected barrier permeability, most likely by opening the tight junctions, only LPS caused cell damage, reflected by increased LDH concentrations in cell culture medium. These results prove a pathogen-specific loss of blood–milk barrier integrity during mastitis, which is characterized by tight junction opening by both LPS and LTA and by additional epithelial cell destruction through LPS.
Key words: mammary, mastitis, blood–milk barrier, tight junction

Short Communication

In the bovine mammary gland, the milk-producing mammary epithelial cells lining the alveoli are involved in the formation of the blood–milk barrier that prevents uncontrolled intermixture of blood and milk (Nguyen and Neville, 1998). Intramammary infections, besides specific physiological stages of the mammary gland like lactogenesis and involution, lead to reduced blood–milk barrier integrity (Linzell and Peaker, 1972; Nguyen and Neville, 1998). This reduction of blood–milk barrier integrity increases the appearance of blood-derived substances in the milk (Stelwagen, 2001; Wheeler et al., 2007; Lehmann et al., 2013; Stelwagen and Singh, 2014). Consequently, the composition of mastitic milk is considerably changed: as well as increased SCC, other blood components such as serum albumin or IgG_2 antibodies can be detected (Burton and Erskine, 2003; Wellnitz et al., 2013). These changes can reduce the quality of milk enormously but also support the innate immune system, as the increased appearance of antibodies in milk can opsonize invaded pathogens for neutrophil phagocytosis (Burton and Erskine, 2003).

Alteration of the blood–milk barrier is caused, at least in part, by an opening of the tight junctions (Burton and Erskine, 2003). Tight junctions seal adjacent mammary epithelial cells, preventing paracellular transport of ions and small molecules. Furthermore, they establish cell polarity by dividing the plasma membrane into apical and basolateral domains (Schneeberger and Lynch, 1992; Stevenson and Keon, 1998).

The loss of blood–milk barrier integrity during mastitis was observed to be pathogen-specific: experimentally induced mastitis by *Escherichia coli*-derived LPS resulted in a more pronounced transfer of blood components, including IgG_2, into milk than mastitis induced

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by lipoteichoic acid (**LTA**) from *Staphylococcus aureus*, even though the increase of SCC was similar (Wellnitz et al., 2013). A differential transfer of blood components induced by different pathogens could influence the course and the healing process of mastitis. Therefore, the objective of the present study was to investigate the pathogen-specific mechanisms of the impairment of the blood–milk barrier during mastitis. In an in vitro model of bovine mammary epithelial cells, barrier integrity and tight junction formation was established. The effects of the bacterial cell wall components LPS and LTA from the mastitis pathogens *Escherichia coli* and *Staphylococcus aureus*, respectively, on epithelial barrier integrity and cytotoxicity were investigated.

Primary bovine mammary epithelial cells (**pbMEC**) of 3 lactating Holstein dairy cows with clinically healthy udders (SCC $<10^5$ cells/mL) were isolated directly after slaughter and cryopreserved in the first passage as previously described (Wellnitz and Kerr, 2004). Cells of all 3 cows were seeded separately at concentrations of 10^5 cells/cm^2 onto 0.4-μm Transwell polyester membrane inserts in 12-well cell culture plates (Corning Inc., Corning, NY). Cells were grown in 1.5 and 0.5 mL of growth medium on the basolateral and apical sides, respectively, for 4 to 5 d in the presence of 5% CO_2 at 37°C. Medium was changed every 48 h.

To visualize the cell contacts by electron microscopy, pbMEC grown on Transwell inserts were washed with PBS and fixed in 2.5% glutaraldehyde in 0.15 *M* HEPES buffer (707 mOsm, pH 7.4) at 4°C for several days. Cells were then washed with 0.15 *M* HEPES buffer and fixed for 1 h in 1% OsO_4 in 0.1 *M* sodium cacodylate buffer (total osmolarity: 369 mOsm, pH 7.4). After washing with 0.05 *M* maleate-NaOH buffer (pH 5.0), the membranes were dehydrated in 70, 80, and 96% ethanol each for 15 min at room temperature. Subsequently, cells were immersed in 100% ethanol 3 times for 15 min, incubated in ethanol-epon (1:1) overnight at room temperature, embedded in epon, and left to polymerize at 60°C for 4 d. Ultrathin sections (70 nm) were cut on a Reichert-Jung Ultracut E microtome (Leica Microsystems, Vienna, Austria), mounted on polyvinyl formal (formvar)-coated 2- × 1-mm single-slot copper grids, and double-stained with 1% uranyl acetate and 3% lead citrate. Transmission electron microscopy (EM 400, Philips, Eindhoven, the Netherlands) indicated that the pbMEC displayed specific structural features of epithelial tissues (Figure 1): a continuous layer of cells was formed with apico-basal polarity. The basal membrane was attached to the Transwell membrane. Microvilli were formed on the luminal cell surface, and intercellular junctions were visible between neighboring cells. As described previously in mammary cells (Pickett et al., 1975), these connections include desmosomes and tight junctions.

To confirm the existence of tight junctions between adjacent cells, pbMEC of all 3 cows were separately seeded on collagen-coated coverslips and cultured until confluency. Immunofluorescent labeling of the tight junction protein zona occludens-1 (**ZO-1**) in pbMEC grown on coverslips was performed as described previously (Hernandez et al., 2011) using a monoclonal mouse anti-human, anti-dog ZO-1 antibody (Molecular Probes/Thermo Fisher Scientific, Waltham, MA). Visualization using a fluorescence microscope (BX41, Olympus, Center Valley, PA) showed that ZO-1 was expressed at the intercellular contacts of pbMEC (Figure 2).

After confirmation of cell connections, the barrier function of the pbMEC on Transwells was evaluated by 2 complementary assays: transepithelial electrical resistance (**TEER**) and diffusion of the fluorescent dye Lucifer yellow (**LY**) across confluent layers of pbMEC. For measurement of TEER, an epithelial volt-ohmmeter (World Precision Instruments, Sarasota, FL) was used as described by Ma et al. (1999). The TEER value is inversely proportional to the permeability, and it represents a marker for intercellular tight junction integrity (Berkes et al., 2003). The mean of 3 Transwell inserts with pbMEC successively measured 3 times resulted in a TEER value of 1,531 ± 75 Ωcm^2 and that of a Transwell insert without any cells was 135 ± 7 Ωcm^2, demonstrating that primary pbMEC from different cows cultured on Transwell inserts were able to form a cell layer with tight intercellular connections.

To determine dynamic changes in barrier integrity in response to different mastitis pathogens, we challenged cells with LPS and LTA. Cells of 3 cows were cultured separately on Transwell inserts and grown for 4 to 5 d until they formed tight cell layers, confirmed by a TEER >1,500 Ωcm^2. Before endotoxin treatment, inserts were washed twice with Dulbecco's PBS (Sigma-Aldrich, Buchs, Switzerland), and the growth medium was replaced in both chambers. Then, 0.2 or 0.5 mg/mL LPS or 2 or 20 mg/mL LTA (Sigma-Aldrich) was added to the upper chamber. The permeability of the epithelial barrier was detected by paracellular diffusion of LY in the following manner: 0.5 mL of 50 μ*M* LY (Lucifer yellow CH lithium salt; Biotium, Hayward, CA), suspended in phenol red–free Dulbecco's modified Eagle's medium supplemented with 2.5 m*M* L-glutamine (Sigma-Aldrich) and 5% fetal bovine serum (LY assay medium), was added to the apical compartment of Transwell inserts, and 1.5 mL of LY assay medium alone was added to the basolateral compartment. The paracellular diffusion of membrane-