J. Dairy Sci. 101:1–10
https://doi.org/10.3168/jds.2017-14312

Selected reaction monitoring mass spectrometry of mastitis milk reveals pathogen-specific regulation of bovine host response proteins

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ABSTRACT

Mastitis is a major challenge to bovine health. The detection of sensitive markers for mastitis in dairy herds is of great demand. Suitable biomarkers should be measurable in milk and should report pathogen-specific changes at an early stage to support earlier diagnosis and more efficient treatment. However, the identification of sensitive biomarkers in milk has remained a challenge, in part due to their relatively low concentration in milk. In the present study, we used a selected reaction monitoring (SRM) mass spectrometry approach, which allowed the absolute quantitation of 13 host response proteins in milk for the first time. These proteins were measured over a 54-h period upon an in vivo challenge with cell wall components from either gram-negative (lipopolysaccharide from *Escherichia coli*; LPS) or gram-positive bacteria (peptidoglycan from *Staphylococcus aureus*; PGN). Whereas our data clearly demonstrate that all challenged animals have consistent upregulation of innate immune response proteins after both LPS and PGN challenge, the data also reveal clearly that LPS challenge unleashes faster and shows a more intense host response compared with PGN challenge. Biomarker candidates that may distinguish between gram-negative and gram-positive bacteria include α-2 macroglobulin, α-1 antitrypsin, haptoglobin, serum amyloid A3, cluster of differentiation 14, calgranulin B, cathepsin C, vanin-1, galectin 1, galectin 3, and IL-8. Our approach can support further studies of large cohorts of animals with natural occurring mastitis, to validate the relevance of these suggested biomarkers in dairy production.

Key words: mastitis, selected reaction monitoring, host response proteins, lipopolysaccharide, peptidoglycan

INTRODUCTION

Mastitis, an inflammatory reaction of the mammary gland, represents a major challenge to animal health in the dairy farming industry, compromising both animal welfare and economic gain (Hogeveen et al., 2011). *Escherichia coli* and *Staphylococcus aureus* are 2 of the major mastitis-causing pathogens in the mammary gland, leading different progressions of pathology (Bannerman, 2009; Schukken et al., 2011; Wall et al., 2016a). Mastitis caused by *E. coli* is usually associated with acute inflammation and severe clinical symptoms, whereas *Staph. aureus* infections mainly result in chronic and subclinical infections (Yang et al., 2008; Hernández-Castellano et al., 2017a). The innate immune system responds to the earliest stages of the infection and relies on cell surface receptors that recognize conserved microbial molecules (Schukken et al., 2011) as well as soluble components, such as acute phase proteins and cytokines, which are secreted into blood and milk (Aitken et al., 2011).

Regulation of bovine host response proteins is not fully understood, but accumulating evidence suggests that host response is specifically tailored to different types of mastitis-causing pathogens (Bannerman, 2009; Griesbeck-Zilch et al., 2008; Yang et al., 2008). Hence, the abundance of specific host response proteins secreted in milk may provide information for early monitoring of mastitis pathogens. Validated markers could be integrated in automated milking systems to provide routine surveillance of animal health and help to improve detection, diagnosis, and treatment of mastitis in dairy herds. Nonetheless, accurate quantification of specific proteins in milk is still technically challenging. Shotgun proteomics has been applied to identify mastitis biomarkers in bovine milk (Danielsen et al., 2010; Boehmer, 2011); however, these studies have failed to report less-abundant proteins related to the immune response, such as cytokines and their receptors. This is partly due to the stochastic nature of the precursor selection in shotgun MS, which is bi-

Received December 19, 2017.
Accepted February 16, 2018.
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ased toward the more-abundant proteins in complex biological samples such as milk, blood, and tissues (Domon and Aebersold, 2010; Hernández-Castellano et al., 2014, 2015). Selected reaction monitoring (**SRM**) by MS was developed to provide increased sensitivity, dynamic range, and reproducibility of protein analyses (Picotti and Aebersold, 2012). The SRM approach provides absolute quantification of selected protein-specific peptides by paired correlation to stable isotope-labeled standards and is currently the method of choice for sub-femtomolar quantification of specific protein biomarkers across diverse biological samples, including mammalian tissues and body fluids (Ebhardt et al., 2015; Hernández-Castellano et al., 2016; Kusebauch et al., 2016).

Lipopolysaccharides have been used in several studies to mimic intramammary *E. coli* mastitis in vivo as well as in cultured mammary epithelial cells (Burvenich et al., 2003; Vels et al., 2009; Wall et al., 2016b). Similarly, peptidoglycans (**PGN**) have been used to mimic intramammary *Staph. aureus* mastitis, but so far only in cultured bovine mammary epithelial cells (Mount et al., 2009; Im et al., 2014; Sulabh et al., 2016) and the effect of PGN on expression of host response proteins in live animals remains uncharacterized. Based on these considerations, the aim of our study was to investigate a time-resolved and parallel monitoring of bovine host response to LPS and PGN, measured by the absolute quantity and concentration dynamics of 13 specific host response proteins in milk.

MATERIALS AND METHODS

Animals and Treatments

Six healthy Danish Holstein-Friesian cows in mid-lactation were randomly selected for 2 parallel intramammary challenge experiments. Three cows were inoculated with LPS (LPS O111:B4, Sigma Aldrich A/S, Brøndby, Denmark) and 3 cows were similarly inoculated with PGN (Sigma Aldrich A/S). Infusions were performed in one quarter with 10 mL of sterile 0.9% NaCl containing either 20 μg of LPS or PGN per milliliter (total dose of 200 μg) 3 h after the morning milking following procedures described in Vels et al. (2009). Before the infusions, milk SCC were measured in all quarters, resulting in SCC <100,000 cells/mL. The SCC was determined directly upon sampling according to Fogsgaard et al. (2012) and Torres et al. (2014) using a DeLaval somatic cell counter (DeLaval International AB, Tumba, Sweden).

All procedures and experimental handling of animals were approved by the Danish Animal Experiments Inspectorate (license no 210601–075, J.no. 2001/561–410) and conducted in the dairy barn facilities at the Department of Animal Science, Aarhus University.

Milk Samples

Milk samples were collected from the infused quarter as well as from an unchallenged, negative control quarter at 6 different time points from each cow: 0, 4, 8, 12, 24, and 54 h after LPS and PGN inoculations. The SCC was determined directly upon sampling as described above. Milk samples were filtered using a 100-μm nylon mesh (Sefar AG, Thal, Switzerland) cut to fit a 6-cm polystyrene funnel and stored at −20°C until further processing. Milk samples were thawed at 37°C to ensure homogenization of milk fat. The casein proteins were precipitated by adjusting the pH to 4.6 by adding 0.06% trifluoroacetic acid (**TFA**) to the milk (9:1), followed by centrifugation at 15,000 × *g* for 10 min at 10°C. After that, the milk whey fraction (supernatant) was collected for further analysis. Protein concentration was determined using the Pierce BCA Protein Kit (VWR-Bie & Berntsen A/S, Herlev, Denmark) with BSA as standard, according to manufacturer's instructions.

Digestion of Milk Proteins

Each whey sample (200 μg of protein) was precipitated using ice-cold acetone and resuspended in 19 μL of digestion buffer (0.5 *M* triethylammonium bicarbonate, 0.1% vol/vol SDS). Protein disulfide bonds were reduced with 1 μL of 50 m*M* Tris (2-carboxyethyl) phosphine hydrochloride to reach a final concentration of 2.5 m*M* Tris (2-carboxyethyl)phosphine hydrochloride and incubated at 60°C for 1 h. Proteins were then alkylated by adding 1 μL of 200 m*M* iodoacetic acid and samples were incubated at room temperature for 10 min in the dark. Proteins were digested with trypsin (1:10 wt/wt; Sciex, Framingham, MA) at 37°C for 16 h. Samples were passed through a centrifugal filter (pore size 0.2 μm; VWR International, West Chester, PA) for 10 min at 10,000 × *g* at 10°C and dried down in a vacuum centrifuge.

Mixed-Mode Cation Exchange Purification of Milk Peptides for SRM Analysis

Dried peptides (50 μg) were dissolved in 2% (vol/vol) acetonitrile (**ACN**), 0.1% (vol/vol) formic acid (pH <3) and purified with mixed-mode ion-exchange. An Oasis MCX μElution Plate (Waters, Milford, MA) was preconditioned with 800 μL of methanol and equilibrated with 3 × 800 μL of 0.1% (vol/vol) TFA in water before peptide samples were loaded on the plate, fol-