Journal of Dairy Research (2005) **72** Special Issue 120–124. © Proprietors of Journal of Dairy Research 2005
doi:10.1017/S0022029905001159 Printed in the United Kingdom

Gene expression of factors related to the immune reaction in response to intramammary *Escherichia coli* lipopolysaccharide challenge

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Pathogenic microorganisms invading the mammary gland induce an inflammatory reaction which includes an increase of somatic cells in milk and activation of bacteriostatic enzymes and proteins in milk. During spontaneously occurring subclinical mastitis the somatic milk cells, mainly macrophages, secrete cytokines, eicosanoids, acute phase proteins and other immunomediators. In contrast, the bacteriostatic protein lactoferrin is mainly secreted by mammary epithelial tissue, while major milk proteins like α-lactalbumin and κ-casein are down-regulated already during subclinical infection.

Changes of the mRNA expression of various immunomediators in the mammary tissue of cows during 12 h after induction of mastitis *via* intramammary administration of lipopolysaccharide (LPS) in several studies are reported. Six healthy lactating cows were injected in one quarter with 100 μg *Escherichia coli*-LPS (O26 : B6) and the contralateral quarter with saline (9 g/l) serving as control. mRNA expression in mammary biopsy samples of various inflammatory factors and milk proteins at 0, 3, 6, 9 and 12 h after LPS administration was quantified by real-time reverse transcription-PCR.

In LPS-challenged quarters tumour necrosis factor α and cyclooxygenase-2 mRNA expression increased to their highest values ($P<0{\cdot}05$) at 3 h after LPS-challenge. Expression of lactoferrin, lysozyme, inducible nitric oxide synthase, and of the apoptotic factors caspase-3, caspase-7 and FAS was elevated ($P<0{\cdot}05$) and peaked at 6 h after challenge. No significant increase in mRNA expression of platelet-activating factor acethylhydrolase, 5-lipoxygenase, and insulin-like growth factor 1 was found. None of the parameters tested did change significantly in the control quarters. mRNA expression of major milk proteins did not change significantly in response to the LPS challenge (α_{S1}-casein, α_{S2}-CN, β-CN and β-lactoglobulin) except for α-lactalbumin which decreased ($P<0{\cdot}05$) in LPS-treated and control quarters and for κ-CN which decreased in the LPS-treated quarters. In conclusion, mRNA expression of the majority albeit not all inflammatory factors changed within hours of LPS challenge. Decreased gene expression of α-lactalbumin and κ-CN may reduce milk yield and suitability for cheese production.

Keywords: lipopolysaccharide, mastitis, mRNA, immunology.

A cow's udder is protected against pathogenic microorganisms by a variety of defence mechanisms. Besides an anatomical-histological barrier in the teat canal (the main entrance pathway of pathogens) bactericidal and bacteriolytic enzymes and proteins, the unspecific cellular immune response is considered as the crucial component of the defence against pathogens.

A fast and efficient reaction of the involved defence systems is necessary to allow an immediate immune reaction and to inhibit microbial growth in the mammary gland. Antimicrobial enzymes and proteins have to be synthesized and activated, and, most importantly, an efficient transfer of leukocytes from the blood into the mammary tissue and milk must be elicited as soon as possible after pathogen invasion. The main leukocyte populations in milk are macrophages, lymphocytes and polymorphonuclear neutrophil granulocytes (PMNs) (Fox et al. 1985; Sarikaya et al. 2004). In healthy quarters with low concentration of immune cells in the milk (somatic cell count; SCC) the predominant cell population are macrophages. In contrast, during augmented immune

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reaction and inflammation the SCC markedly increases and concomitantly the PMN fraction becomes the predominant cell population reaching close to 100% during a severe clinical mastitis. It has been shown on the basis of quantitative mRNA analysis that immunologically important factors like cytokines and other inflammatory mediators are expressed by both the mammary tissue and the immune cells (Pfaffl et al. 2003). However, the level of expression varies considerably between both sources. While cytokines and lipid mediators are mainly expressed by immune cells, antimicrobial defence proteins like lactoferrin originate mainly from the mammary tissue (Pfaffl et al. 2003). Within the somatic milk cells, the cytokine and mediator expression levels were much higher in the macrophage than in the PMN fraction (Wittmann et al. 2002) thus underlining the central importance of macrophages for the initiation of an immune response. Contrary to proteins related to the immune defence, the synthesis of the major milk proteins is diminished during inflammation (Schmitz et al. 2004a, b).

A number of studies have been conducted to investigate short-term changes of various inflammatory factors and milk proteins during 12 h from an experimental mastitis induced by *Escherichia coli* endotoxin (lipopolysaccharide, LPS). All analyses were based on the determination of mRNA of the respective factors or key enzymes of their metabolism using quantitative real-time reverse transcription-PCR. This allowed the analysis of various factors although only a small amount of tissue (30–60 mg) was available from repeated biopsy samples of mammary tissue of one LPS-treated and one control quarter every 3 h.

Experimental procedures

The experiment was performed with six dairy cows in their first to fourth lactation, which were free of intramammary inflammation. Only quarters with a SCC<150 000 cells/ml and milk samples that cultured negative for mastitic pathogens were accepted for the study. Cows were intramammary injected in one quarter (LPS quarter) with 100 μg *Esch. coli*-lipopolysaccharide (Serotype O26 : B6; Sigma Chem. Co., St. Louis, USA) in 10 ml saline and in the contralateral quarter (control quarter) with 10 ml saline (9 g/l) through the teat canal immediately after morning milking. Mammary biopsy samples of both quarters were taken immediately before (0 h) and at 3, 6, 9 and 12 h after injection of LPS. Biopsy samples of 30–60 mg were taken with a human Bard® Magnum® Biopsy instrument (BARD, Covington, GA) and a Core Tissue Biopsy Needle (12 g × 10 cm) (BARD, Covington, GA).

Total RNA of mammary biopsy samples was isolated. Synthesis of first strand complementary DNA (cDNA) was performed with reverse transcriptase (MMLV-RT, Promega, Madison, WI, USA) and random hexamer primers (MBI Fermentas, St. Leon-Rot, Germany). Amplification of target nucleic acids was accomplished by real-time polymerase chain reaction (PCR) in a LightCycler (Roche Diagnostics, Mannheim, Germany).

Cytokines and other molecules involved in blood leukocyte invasion

A number of cytokines, lipid mediators and other factors are responsible for augmented leukocyte influx from blood into milk. The mRNA expression encoding for tumour necrosis factor-α (TNF-α), one of the most important pro-inflammatory cytokines (Watanabe et al. 2000), increased transiently ($P<0{\cdot}05$) in LPS-challenged quarters to reach highest values at 3 h after LPS challenge (Schmitz et al. 2004a). Highest levels were about 47-fold above baseline. Thereafter, TNF-α mRNA showed a steady decrease. TNF-α increased also in the control quarters, albeit only slightly, most likely in response to the biopsy treatment. This indicates the highly sensitive reaction of TNF-α in response to minimal stimuli. TNF-α plays a key role in eliciting the acute phase response following bacterial invasion of the udder, including accumulation of leukocytes at the site of infection (Riollet et al. 2000). Recombinant pro-inflammatory like TNF-α increase the rate of mammary gland involution and concomitantly the lactoferrin concentrations in milk (Wedlock et al. 2004). Possibly, the activation of apoptotic enzymes during inflammation (Didier & Bruckmaier, 2004) comes along with a down-regulation of milk proteins and simultaneous up-regulation of lactoferrin (Schmitz et al. 2004a, b). TNF-α has been demonstrated to be involved in iNOS up-regulation in response to LPS challenge (Kleinert et al. 2003).

Besides cytokines, also lipid derived mediators such as prostaglandins and leukotrienes are involved in the immune response of the mammary gland (Wittmann et al. 2002). In our study, the mRNA expression of the key enzyme of prostaglandin biosynthesis, cyclooxygenase-2 (COX-2), was transiently increased and peaked at 3 h after LPS challenge (Schmitz et al. 2004a). In contrast, there was no significant change of key enzymes related to platelet activating factor (PAF-AH) or leukotriene (5-LO) metabolism within the 12 h of our experiment. Possibly some of these factors which do not belong to the class of the pro-inflammatory mediators, would react after this period in a true infection scenario.

Insulin-like growth factor-I (IGF-I) mRNA expression did not change significantly in response to LPS (Schmitz et al. 2004a). This finding is surprising because in a previous investigation IGF-I protein levels increased about 3-fold in the milk of LPS-treated quarters (Bruckmaier et al. 1993). However, in this earlier study the LPS dosage was about 10-fold higher than in the recent study by Schmitz et al. (2004a). Possibly, the elevated IGF-I concentration in response to LPS challenge was due to an increased paracellular transfer of IGF-I from blood into milk *via* leaky tight junctions, i.e. on a similar basis as the concentrations

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of sodium and chloride ions are regulated (Bruckmaier et al. 2004).

Induction of mRNA expression encoding for antibacterial proteins and enzymes

The mRNA expression of lactoferrin (Lf), lysozyme (Lz) and inducible nitric oxide synthase (iNOS) increased significantly within 3 h after LPS injection and peaked at 6 h after challenge. For Lz, a significant, though, in comparison with the LPS quarter, small rise of mRNA expression was also observed in the control quarter (Schmitz et al. 2004a). Lf is known to increase in bovine milk during clinical mastitis (Harmon et al. 1976; Kawai et al. 1999). Increased concentration of Lf in the mammary secretion during mastitis or involution indicates that the regulation of Lf in the mammary gland is contrary to that of other milk proteins (Schanbacher et al. 1993). These changes were paralleled by changes in Lf mRNA expression (Neville & Zhang, 2000). Besides Lf secreted by the secretory epithelium (Persson et al. 1992; Pfaffl et al. 2003; Schmitz et al. 2004a) Lf is also released by PMNs during inflammation (Harmon & Newbould, 1980). Lz is of relevance to the natural defence system of the mammary gland due to its bacteriostatic and even bactericidal effects on udder pathogens (Lunau, 1989). In several studies Lz concentration was low in normal bovine milk but increased during mastitis (Carlsson et al. 1989; Persson et al. 1992). A significant correlation between concentrations of Lz and SCC was also observed (Götze et al. 1977; Persson et al. 1992). Persson et al. (1992) and Steinhoff et al. (1994) concluded that leukocytes are the most likely source of Lz during inflammation whereas an important contribution of the mammary epithelial cells in Lz synthesis is most likely according to Schmitz et al. (2004a). Intramammary infusion of LPS caused an enhanced intramammary production of nitric oxide (NO) (Bouchard et al. 1999; Blum et al. 2000). In accordance LPS challenge augmented iNOS mRNA expression (Schmitz et al. 2004a). Activated macrophages synthesize NO to eliminate intracellular pathogens. Therefore NO plays a key role in mediating microbistatic or microbicidal activity (Jungi, 2000). NO synthesis is catalyzed by iNOS which is known to be induced by LPS. TNF-α is supposedly responsible for iNOS up-regulation and hence increased NO production (Blum et al. 2000; Kleinert et al. 2003). This assumption is supported by mRNA expression data (Schmitz et al. 2004a) showing a peak of mRNA expression encoding for TNF-α and the transcription factor NF-κB clearly before the peak of iNOS mRNA expression. NF-κB mRNA expression increased about 8-fold within 3 h in response to LPS challenge and gradually decreased thereafter in our study. NF-κB plays a crucial role, possibly together with other transcription factors such as STAT-1α, in the synthesis of iNOS because it is a central target of activators or inhibitors of iNOS expression such as LPS, TNF-α, and IL-1β (Kleinert et al. 2003).

Factors related to apoptosis

Toxins such as LPS may induce apoptosis in both milk cells and mammary tissue. Apoptosis is characterized by a defined cascade of morphological and biochemical events finally leading to the controlled death and removal of the respective cells without any signs of inflammation. TNFα, as released in response to LPS challenge, may be one of the factors that induce apoptosis (Maianski et al. 2003). A major event during the course of apoptosis is the activation of caspases that contribute to cleavage of nuclear proteins and DNA fragmentation (Robertson et al. 2000). Caspase-3 and caspase-7 belong to the group of effector caspases. Caspase-3 participates in bone marrow derived neutrophil apoptosis (Woo et al. 1998). In response to intramammary LPS infusion caspase-3 and caspase-7 mRNA expression increased dramatically till 6 h from the start of infusion and thereafter decreased again. In addition, FAS mRNA expression increased with a peak at 3 h from infusion of LPS (Didier & Bruckmaier, 2004). Although caspases are stored as pro-enzymes and activated by cleavage of the pro-domain in response to a 'death signal' (Zhivotovsky et al. 1999) there is obviously also a short-term up-regulation of the mRNA of these factors. LPS induces apoptosis in neutrophils (Van Oostveldt et al. 2002) but also in mammary secretory tissue cells (Didier & Bruckmaier, 2004). The activation of mammary epithelial cells by LPS is possibly mediated *via* soluble CD14 (Wang et al. 2002). It is tempting to speculate that mammary epithelial cells undergoing programmed cell death in response to bacterial toxins are changing their protein expression pattern during early stages of apoptosis. This could cause the observed decline of synthesis of milk proteins like α-lactalbumin and κ-casein while concomitantly the synthesis of antibacterial proteins and enzymes like Lf and Lz is dramatically increased (Schmitz et al. 2004a).

Acute phase proteins

Acute phase proteins are important regulators of the immune system and have stimulatory effects in the early stage of immune response. Serum amyloid A (SAA) and haptoglobin were shown to be increased in serum and milk during acute *Staphylococcus aureus* mastitis, SAA also in chronic cases (Grönlund et al. 2003).

Changes of mRNA expression encoding for haptoglobin representing acute phase proteins were investigated in response to LPS challenge (Hiss et al. 2004). A clear increase of mRNA expression in the LPS quarters was found. SAA may play an important role in leukocyte recruitment in the mammary gland as it has chemotactic effects on leukocytes (Badolato et al. 1994). Its expression is dramatically increased in mammary epithelial cells in response to *Esch. coli* LPS treatment or *Staph. aureus* infection (Wellnitz & Kerr, 2004).

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Milk proteins

α_{S1}-Casein-, α_{S2}-CN-, β-CN- and β-lactoglobulin-mRNA expression did not change significantly albeit their values were numerically lower at 9 h after LPS administration as compared with 0 h. A significant decrease of mRNA expression relative to time 0 at 9 h post inoculation was observed for α-lactalbumin in both, LPS and control quarter, and for κ-CN in the LPS quarter (Schmitz et al. 2004a, b). This effect may be caused by early apoptotic reactions of the mammary epithelial cells (Didier & Bruckmaier, 2004), i.e. proteins which determine milk yield or casein micelle formation, respectively, are down-regulated while simultaneously proteins involved in the immune response like Lf and Lz are up-regulated.

Conclusions

In conclusion, the quantitative measurement of mRNA expression of inflammatory factors is useful to determine the reaction of a number of factors simultaneously in small biopsy tissue samples. The gene expression of most inflammatory factors increased within hours after LPS challenge. Decreased gene expression of α-lactalbumin in response to LPS explains the immediate milk loss even during subclinical mastitis. Decreased κ-casein could be related to changed structures of the casein micelles in milk and hence potentially a reduced suitability of mastitis milk for cheese production.

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