Short communication: *Staphylococcus aureus* infection modulates expression of drug transporters and inflammatory biomarkers in mouse mammary gland

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ABSTRACT

Mastitis is the most common disease in dairy herds worldwide and is often caused by *Staphylococcus aureus*. Little is known about the effect of mastitis on transporters in the mammary gland and the effect on transporter-mediated secretion of drugs into milk. We studied gene expressions of ATP-binding cassette and solute carrier transporters in *S. aureus*-infected mammary glands of mice. On d 7 of lactation, NMRI mice were inoculated with 1,000 cfu of *S. aureus* in 2 mammary glands and with a saline vehicle in 2 control glands. Gene expression of the transporters, *Bcrp*, *Mdr1*, *Mrp1*, *Oatp1a5*, *Octn1*, and *Oct1*, and of *Csn2*, the gene encoding β -casein, were determined in mammary glands at 72 h after treatment. As biomarkers of the inflammatory response gene, expressions of the cytokines *Il6*, *Tnfa*, and the chemokine *Cxcl2* were measured. Despite a high individual variation between the 6 animals, some characteristic patterns were evident. The 3 inflammatory biomarkers were upregulated in all animals; *Csn2* was downregulated compared with controls in all animals, although not statistically significantly. Both *Mrp1* and *Oatp1a5* were statistically significantly upregulated and *Bcrp* was downregulated. Gene expression of *Bcrp* followed the expression of *Csn2* in each of the animals, indicating a possible co-regulation. The findings demonstrate that *S. aureus* infection has an effect on expression of drug transporters in the mammary gland, which may affect secretion of drugs into milk and efficacy of drug therapy.

Key words: mastitis, drug transporters, mammary gland, *Staphylococcus aureus*

Short Communication

Staphylococcus aureus is recognized to be a major contagious mammary pathogen, causing clinical as well

as subclinical mastitis in cattle (Bradley, 2002; Roy and Keefe, 2012; Royster and Wagner, 2015); it frequently gives rise to persistent and chronic infections (Degen et al., 2015) and, hence, significant economic loss for dairy producers (Bradley, 2002). Drug transporters belonging to the ATP-binding cassette (ABC) and solute carrier (SLC) superfamilies are membrane proteins mediating active or facilitated cellular influx and efflux transport of endogenous compounds, numerous drugs, and other chemicals (Sai and Tsuji, 2004; Klaassen and Aleksunes, 2010; Schuetz et al., 2014; Chen et al., 2016). Individual members of the superfamilies, such as breast cancer resistance protein (BCRP/ABCG2), multidrug resistance protein 1 (MDR1/ABCB1), multidrug resistance-associated protein 1 (MRP1/ABCC1), solute carrier organic anion transporter family member 1 A2 (OATP1A2/SLCO1A2; the bovine ortholog to the murine *Oatp1a5*), organic cation transporter novel protein type 1 (OCTN1/SLC22A4), and organic cation transporter 1 (OCT1/SLC22A1), are expressed differentially during the pregnancy-lactation cycle in mammary epithelial cells of cattle (Farke et al., 2008; Mani et al., 2009), mice and rats (Gilchrist and Alcorn, 2010; Lamhonwah et al., 2011; Ito et al., 2014; Yagdiran et al., 2016), and humans (Alcorn et al., 2002; Jonker et al., 2005). Emerging evidence has demonstrated that infection and inflammation in various tissues affect the expression and function of transporters with impact on drug distribution and efficacy of therapy (Petrovic et al., 2007; Martinez et al., 2008; Morgan et al., 2008; Cressman et al., 2012, 2014; Gandhi et al., 2012). Reported data are mainly from liver, intestine, kidney, brain, and placenta, and little is known about the effect of inflammation in the mammary gland on the expression of transporters. This is surprising due to the high incidence of mastitis in dairy herds; likewise, the potential effect of inflammation on drug transporters could have an effect on milk quality, by modulating excretion of nutrients and toxic compounds into milk, and on the therapeutic efficacy of drugs, which are ligands of the transporters. In the present study, we tested the hypothesis that *S. aureus*-induced mammary inflam-

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mation might affect gene expression of ABC and SLC transporters in the mouse mammary gland.

We used a mouse model of *S. aureus*-induced mammary inflammation to study the effect of gene expression of ABC and SLC transporters, known to vary with lactation stage. As an indicator of milk protein synthesis, expression of *Csn2* (the gene encoding β -casein) was measured, and, as biomarkers of inflammatory response, the proinflammatory cytokines *Il6* and *Tnfa* and the chemokine *Cxcl2* were determined. The procedure for inducing mouse mammary inflammation by a *S. aureus* challenge has been described by Nazemi et al. (2014). Our experiment was performed according to the guidelines of the European Convention for protection of Vertebrate Animals and Animal Experimentation Act under Danish national legislation (Miljø- og Fødevarerministeriet, 2013) and conducted at Technical University of Denmark (Lyngby, Denmark; license number: 2012–15–2934–00587). Briefly, 6 lactating NMRI mice were treated under anesthesia with *S. aureus* on d 7 ± 2 of lactation (10 pups/litter). In each mouse, the fourth and fifth inguinal glands on one side were infused with 100 μ L of a *S. aureus* AO35 (10,000 cfu/mL) suspension, whereas the 2 contralateral inguinal glands were infused with 100 μ L of a physiological saline vehicle. Following inoculation, mothers were kept separated from pups for 4 h to obtain better colonization of the bacteria. After 72 h, the mice were anaesthetized and euthanized by cervical dislocation and the treated mammary glands were collected, frozen in liquid nitrogen, and stored at 80°C.

To isolate total RNA, frozen mammary glands were cut into smaller pieces of 20 to 25 mg on dry ice. The 2 treated mammary glands from each mouse were pooled, as were the 2 controls, and the tissues were homogenized with Trizol (Life Technologies Europe, Naerum, Denmark) and frozen stainless steel beads (5 mm, cat. no. 69989; Qiagen, Hilden, Germany) with TissueLyser II from Qiagen (cat. no. 85300). After centrifugation at $10,000 \times g$ for 10 min at 4°C, the supernatants were transferred into MaXtract High Density 1.5-mL tubes (cat. no. 129046; Qiagen). Final isolation of RNA was performed by using SV Total RNA isolation system kit from Promega (Nacka, Sweden) according to the guidelines. Quantification of the RNA was performed with the RNA specific Quant-iT RiboGreen-kit according to the manufacturer (Thermo Fisher Scientific, Stockholm, Sweden). Sequences of the primers of transporters and *Csn2* are described by Yagdiran et al. (2016). Gene-specific intron-spanning primers to murine [*Cxcl2* (forward) 5'-TCCAGAGCTTGAGTGTGACG-3 and (reverse) 5'-CTTTGGTTCTTCCGTTGAGG-3; *Il6* (forward) 5'-AGTTGCCTTCTTGGGACTGA-3 and

(reverse) 5'-TCCACGATTTCCCAGAGAAC-3; and *Tnfa* (forward) 5'-AGCCCCCAGTCTGTATCCTT-3 and (reverse) 5'-CTCCCTTTGCAGAACTCAGG-3] were designed by the use of the Genome Browser (University of California, Santa Cruz) and Primer3 software (SimGene.com). The primers were synthesized by Cybergene (Huddinge, Sweden). Quantitative gene expression was examined by real-time quantitative reverse-transcription PCR (RT-qPCR) using a Rotor-Gene 3000 (Corbett Research, Mortlake, Australia) by applying the One-tube QuantiTectSYBR Green RT-PCR Kit (Qiagen Nordic, Sollentuna, Sweden) according to the manufacturer's recommendations. Prior to RT-qPCR analysis, the specificity of all primer pairs were tested on murine mammary RNA and shown to generate specific RT-qPCR products with anticipated amplicon sizes and single melting curve peaks. Final primer concentration for all target genes was 0.4 μ M, and 75 or 150 ng of total RNA was used as template in 12.5- μ L RT-qPCR reactions. Nontemplate controls served as blanks and melting curve analysis was performed for each sample to check the specificity of the obtained PCR products. Expressions of target genes were normalized to the geometric average expression of 3 appropriate reference genes (Bustin et al., 2009). As reference genes in mouse mammary gland (as recommended by Han et al., 2010), we used hypoxanthine-guanine phosphoribosyltransferase (*Hprt*), ribosomal protein L13A (*Rpl13a*), and glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*). Relative quantification of mRNA expressions was performed by comparing the quantification cycle (Cq) between the tissues and treatment groups of cells according to the $2^{-(\Delta\Delta Cq)}$ method (Livak and Schmittgen, 2001); Cq 35 was used as cut-off for limit of detection of gene expression. Fold differences were calculated by setting vehicle controls to 1.

Statistical analyses were performed using Minitab 16 software (Minitab Inc., State College, PA). The results were analyzed by Kruskal-Wallis to detect any significant differences among the treatment groups, followed by Mann-Whitney to examine statistically significant differences between 2 groups. The level of significance was set at $P < 0.05$.

Figure 1 shows individual mRNA expressions of the proinflammatory biomarkers, *Cxcl2*, *Il6*, and *Tnfa*, of *Csn2*, as an indicator of milk protein synthesis, and of the transporters *Bcrp*, *Mdr1*, *Mrp1*, *Oatp1a5*, *Octn1*, and *Oct1* for each of the 6 mice. The results are expressed as the ratio between treated and control glands. We found common patterns in response, even if the difference in response rate was wide between the animals. Each of the animals responded to the infection

Figure 1. Ratios between gene expressions in treated mammary glands and in vehicle-treated control glands of inflammatory biomarkers (*Cxcl2*, *Il6*, and *Tnfa*), *Csn2*, and transporters (*Bcrp*, *Mdr1*, *Mrp1*, *Oatp1a5*, *Octn1*, and *Oct1*) 72 h after treatment with 1,000 cfu of *Staphylococcus aureus* or control vehicle in mammary glands of mice. Normalized gene expressions are presented as ratios in the 6 individual animals. Scales for *Cxcl2* and *Il6* are logarithmic.

with a similar expression profile of the inflammatory biomarkers. One of the mice, number 4, had a very low response, and numbers 2 and 3 had the highest response for all 3 biomarkers. Gene expressions of *Csn2* and *Bcrp* were downregulated in all animals and had almost the same profile, indicating an association and a possible co-regulation. Treatment of animals with *S. aureus* resulted in an upregulation in all animals of *Oatp1a5* and *Mrp1*. Gene expressions of *Mdr1* and *Oct1*

were downregulated in 4 animals and upregulated in 1, and *Octn1* was only induced in 1 of the treated animals (Figure 1). No statistically significant differences in gene expressions of the reference genes in mammary glands of control and *S. aureus*-induced animals were detected.

The overall effects of *S. aureus* treatment compared with vehicle controls on gene expression of transporters and the inflammatory biomarkers in mammary gland

Figure 2. Boxplots with gene expressions of inflammatory biomarkers (*Cxcl2*, *Il6*, and *Tnfa*), *Csn2*, and transporters (*Bcrp*, *Mdr1*, *Mrp1*, *Oatp1a5*, *Octn1*, and *Oct1*) 72 h after treatment of mammary glands of mice with 1,000 cfu of *Staphylococcus aureus* (Staph) or vehicle controls (Vehicle). Normalized gene expressions are presented as boxplots, in which dark horizontal lines represent the median, with the box representing the 25th and 75th percentiles and the whiskers the 5th and 95th percentiles (n = 6). Statistically significant differences as compared with vehicle controls: * $P < 0.05$; ** $P < 0.01$.

are illustrated by boxplots in Figure 2, including results from the statistical analysis. Despite the relatively large interindividual variations, the upregulation of the pro-inflammatory biomarkers was statistically significant, as well as the downregulation of *Bcrp* and upregulation of *Mrp1* and *Oatp1a5*.

Breast cancer resistance protein is an efflux transporter located at the apical membrane and known to be strongly induced in the mammary gland epithelium during pregnancy and lactation, which may lead to concentration of BCRP ligands, such as drugs and carcinogenic food contaminants in milk (Jonker et al., 2005; van Herwaarden and Schinkel, 2006). Breast cancer resistance protein has a wide substrate specificity, and veterinary drugs transported by BCRP include the antimicrobials ciprofloxacin, enrofloxacin, norfloxacin, danofloxacin, nitrofurantoin (Alvarez et al., 2006; Merino et al., 2006; Pulido et al., 2006; Pérez et al., 2009; Real et al., 2011; Mealey, 2012), and sulfoxide metabolite of the anthelmintic drug monepantel (Mahnke et al., 2016).

Few investigations have reported effects of mastitis on elimination of veterinary drugs in milk, but none have investigated effects on drug transporters. Nonsteroidal anti-inflammatory drugs flunixin (Kissell et al., 2015) and carprofen (Lohuis et al., 1991) were reported at significantly higher concentrations in milk from mastitic cows than from control cows. These drugs are

not known to be ligands of efflux transporters, and the increased milk excretion was suggested to be due to increased mammary gland vascular permeability and alteration of the blood-milk barrier. Substances can move into milk either through the transcellular route (uptake across the basolateral membrane, followed by secretion across the apical membrane) or, alternatively, through the paracellular route (movement through leaky tight junctions). Paracellular flux is greatest in very early lactation (and is responsible, in part, for accumulation of immunoglobulins in colostrum), but is also increased during mastitis as part of the local immune defense (Stelwagen and Singh, 2014). However, in contrast to the increased nonsteroidal anti-inflammatory drugs transfer, the BCRP substrates norfloxacin (Gips and Soback, 1999) and ciprofloxacin, the active metabolite of enrofloxacin (Rantala et al., 2002), were found to be excreted into milk in lower concentrations in cows with mastitis compared with healthy animals. This clearly suggests that transcellular mechanisms and membrane transporter proteins are involved. The lower concentrations of BCRP ligands in milk in cows with mastitis may be due to decreased expression of BCRP in the mammary epithelial cells of the animals with inflammation compared with in healthy cows. To our knowledge, this is the first study to show effects on drug transporters in an in vivo mastitis model. Our findings demonstrate that *S. aureus* infection has an effect on expression of

drug transporters in the mammary gland, which may affect secretion of drugs into milk and efficacy of drug therapy.

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