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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 expressions 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*, *Tnfα*, 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 coregulation. 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 Fødevareministeriet, 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 μL of a *S. aureus* AO35 (10,000 cfu/mL) suspension, whereas the 2 contralateral inguinal glands were infused with 100 μL 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 × *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 *Tnfα* (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 synthetized 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-μL 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 *Tnfα*, 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