BRIEF COMMUNICATION: Expression of innate immune response genes in mammary epithelia following stimulation with lipopolysaccharide or *Escherichia coli*.

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INTRODUCTION

The innate immune system is essential for keeping the bovine mammary gland disease free. A number of innate immune response genes are expressed in bovine mammary epithelial cells (bMEC), suggesting it contributes to defence against pathogens (Rainard & Riollet, 2006). These responses may play a key role in determining susceptibility to mastitis. The aim of this project was to investigate the expression in bMEC of specific innate immune genes in response to *Escherichia coli* (*E. coli*) and lipopolysaccharide (LPS), the principal immunostimulatory molecule in Gram negative bacteria. These studies were performed *in vitro* with cultured bMEC (*E. coli*) and *in vivo* with LPS infusion in teat canals of dairy cows. A range of other inflammatory factors and milk proteins have also been surveyed in the *in vivo* model (Schmitz *et al*., 2004).

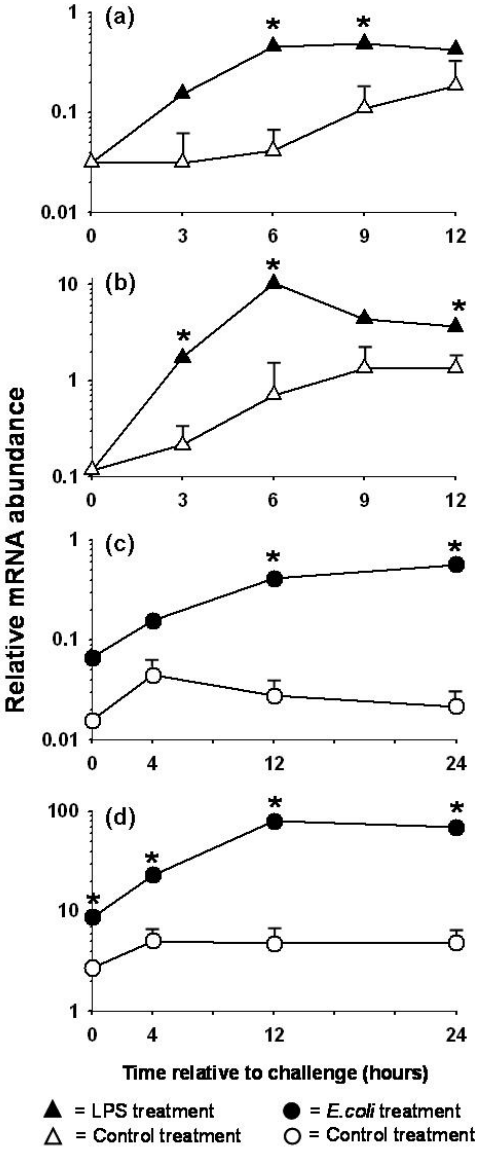
MATERIALS AND METHODS

The *in vivo* experiment using LPS has previously been described (Schmitz *et al*., 2004). Briefly, six healthy lactating dairy cows with a negative culture for mastitic pathogens and a somatic cell count (SCC) of <150,000 cells/mL, were treated with an intramammary infusion of *E. coli*-LPS in one quarter and sterile saline in the contralateral quarter. Mammary biopsies were taken immediately before (0 h) and 3, 6, 9 and 12 hours after infusion. For the *in vitro* experiment using *E. coli*, the primary bMEC were collected from a slaughtered third trimester pregnant cow, digested with collagenase, epithelial cells isolated by Percoll gradient and cryopreserved (B.J. Haigh, Unpublished data). bMEC from a single cow were grown in culture media for 10 days (passaged once), treated with sonicated *E. coli* (ATCC25922, 100ug/ml in phosphate buffered saline (PBS)) or PBS alone. Samples were collected immediately after treatment (0 h) and at 4, 12 and 24 hours (n = 3 wells/time point for both stimulated and control treatments). Total RNA from mammary biopsies and bMEC were extracted, purified and converted to cDNA as described by Schmitz *et al.*, 2004. Quantitative real-time RT-PCR analysis was carried out using the relative standard curve method, with SYBR Green master Mix in the 7900 Sequence Detection System (Applied Biosystems, Foster City, CA) as described by the manufacturer. The mRNA transcripts quantified were angiogenin-1 (ANG1), lipopolysaccharide binding protein (LBP), Mammary serum amyloid A3 (SAA3), RNase4 and statherin (STATH). β-actin was used as an internal control. For the *in vivo* trial, differences in values between treatments at each time were analysed using ANOVA, blocking on animal with 0 h as the covariate. Means adjusted for 0 h values for the treatments at each time point are presented as backtransformed relative mRNA levels with the standard error of difference (SED) between means,. For the *in vitro* experiment a mixed model in GenStat was fitted for values with plate as a random effect and time, treatment and their interaction as fixed effects. Means are presented as backtransformed relative mRNA levels with the SED between means.

RESULTS AND DISCUSSION

In the *in vivo* experiment using LPS, the LAP and SAA3 mRNA levels were increased ($P < 0.05$) in the alveolar tissue of LPS treated quarters when compared to the controls 6 hours after infusion, by 11- and 15-fold, respectively (Figure 1a and 1b). After 6 hours, the difference was reduced, partly due to the elevation of LAP and SAA3 in the untreated quarters, so that at 12 hours the difference was no longer significant for LAP. In contrast, the expression of LBP, ANG1, RNAase4 and STATH was not significantly altered between LPS infused and control quarters. Similar results were observed by stimulating primary bMEC with sonicated *E.coli* extracts. LAP and SAA3 relative abundance was increased ($P < 0.05$) compared to the untreated controls at 12 h by 15- and 17-fold, respectively (Figure 1c and 1d). However, in contrast to the *in vivo* experiment with LPS, the levels of LAP and SAA3 remained significantly elevated compared to the controls until at least 12 hours after treatment. LBP, ANG1 and RNAse4 mRNA levels were unchanged and STATH mRNA could not be detected.

FIGURE 1: Timecourse of changes in mRNA levels of Lingual antimicrobial peptide (a) and (c) and Mammary serum amyloid A (b) and (d) in mammary epithelial cells. mRNA for (a) and (b) were collected following intramammary lipopolysaccharide (LPS) injection and (c) and (d) from *E.coli* stimulated primary cells. * Indicates the control and treated cells are significantly different (P <0.05) at this time point. Error bars show standard error of difference.



Our study investigated the expression of a selection of innate immune genes in the mammary gland. All these genes have a putative role in innate immunity and may potentially protect the mammary gland from infection. However, our data indicate that stimulation with *E.coli* lysates, or the key inflammatory stimuli in *E.coli*, LPS, results in the up-regulation of mRNA expression of only a subset of these genes The genes which are up-regulated, LAP and SAA3, are likely to play a key role in the resolution of the infection. The upregulation of LAP and SAA3 has also been observed in other studies using isolated bMEC stimulated with LPS (Rainard & Riollet, 2006). LAP is known to have antimicrobial properties, with demonstrated effectiveness against several mastitis causing organisms (Selsted *et al*., 1993). SAA3 is a member of the serum amyloid A family which is elevated as part of the acute phase response (Uhlar & Whitehead, 1999). The similar expression profile observed with both the LPS and *E. coli* models suggests that LPS is the key stimulatory molecule in *E. coli* and that bMEC, in the absence of macrophages and monocytes, are able to rapidly detect and respond to the pathogenic stimuli. There are however, some differences in the experiment models. Stimulation of the mammary gland *in vivo* resulted in the increased expression of LAP and SAA3 in the control quarters, possibly reflecting a systemic response to LPS as rectal temperatures were elevated post-infusion (Schmitz *et al.*, 2004), or alternatively local inflammation due to infusion of the saline control. A further difference between the *in vivo* and *in vitro* experiments was the altered mRNA abundance at 0 hours in the cell culture samples. This is likely to be a technical artefact as this effect was seen for all mRNA measured and may have resulted from the *E.coli* extracts. These extracts, which were added at 0 hours contain large amounts of exogenous mRNA, which may have perturbed the real time analysis. This exogenous RNA would have been degraded at later time points.In summary, this study highlights the gene-specific response of the mammary gland towards mastitic pathogens and the utility of the *in vitro* bMEC culture system for analysing innate immune gene regulation. Further investigations of these responses may lead to ways of selecting for cows that are relatively resistant to mastitis.

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REFERENCES

Rainard, P.; Riollet, C. 2006: Innate immunity of the bovine mammary gland. *Veterinary research* **37:** 369-400.

Schmitz, S.; Pfaffl, M.; Meyer, H.H.D.; Bruckmaier, R. 2004: Short-term changes of mRNA expression of various inflammatory factors and milk proteins in mammary tissue during LPS-induced mastitis. *Domestic animal endocrinology* **26:** 111-112.

Selsted, M.; Tang, Y.; Morris, W.; McGuire, P.; Novotny, M.; Smith, W., Henschen A.; Cullor, J. 1993: Purification, primary structures, and antibacterial activities of beta-defensins, a new family of antimicrobial peptides from bovine neutrophils. *Journal of biological chemistry* **268:** 6641-6648.

Uhlar, C.; Whitehead, A. 1999: Serum amyloid A, the major acute-phase reactant. *European journal of biochemistry* **265:** 501-523.