BRIEF COMMUNICATION: Understanding the interaction of prolactin and leukaemia inhibitory factor signalling during the switch from lactation to involution

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INTRODUCTION

The molecular mechanisms involved in involution of the bovine udder, a process which underpins lactation persistency, are poorly understood. During rodent lactation, STAT5 is activated in response to prolactin (PRL) to stimulate milk protein production (Liu *et al.,* 1997). At the onset of involution, STAT5 is rapidly inactivated (Schmitt-Ney *et al*., 1992), and STAT3 is activated by leukaemia inhibitory factor (LIF) to regulate mammary epithelial cell (MEC) apoptosis and tissue remodelling (Kritikou *et al.*, 2003). It is unclear how the reciprocal activation of the two STAT pathways is regulated. Recent evidence suggests the LIF-STAT3 pathway may inhibit PRL-STAT5 signalling via suppressor of cytokine signalling 3 (SOCS3) (Dif *et al.*, 2001; Granillo *et al.*, 2007). The aim of this study was to investigate STAT5/3 signalling at the onset of involution in the bovine mammary gland, and construct a mathematical model to determine if LIF suppresses milk synthesis by inhibiting PRL. Ultimately, this may allow the development of novel strategies for improving milk production especially in the later stages of lactation.

MATERIALS AND METHODS

Involution was induced by abrupt termination of milking in 48 non-pregnant Friesian heifers at mid-lactation (92 ± 3 days in milk with a daily milk yield, 14.3 ± 0.3 kg/cow). Mammary alveolar tissue was obtained from healthy quarters following slaughter at 0, 6, 12, 18, 24, 36, 72 and 192 h (n = 6 per group) after the last milking; and total RNA was extracted, purified and converted to cDNA as described by Singh *et al.*, (2005). SOCS3 mRNA was quantified by real-time reverse transcription polymerase chain reactions (RT-PCR) 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. Ubiquitin was used as an internal control. Western blotting was performed on mammary protein extracts as described by Singh *et al*., (2008) using polyclonal antibodies directed against pSTAT5 (1:30,000, provided by Dr. Wheeler, AgResearch) and pSTAT3 (1:10,000, Santa Cruz Biotechnology, Inc., CA). Immunoreactive bands were subjected to densitometric analyses (GS-800; Bio-Rad Laboratories Pty. Ltd., Auckland). The differences between means were analyzed using ANOVA in the Minitab software package (Minitab Inc., 2003). For SOCS3 mRNA, the means for each group were back-transformed. The SOCS3 mRNA and STAT5/3 protein data are expressed as the fold change ± standard error of mean relative to the 6-h mean. STAT5/3 protein data are expressed as the fold change with the standard error of the difference relative to the 6 h mean. A mathematical model consisting of a set of coupled differential equations was constructed to represent the interaction of the STAT5 and STAT3 signalling pathways. This model was formed by creating sub-models of the PRL-STAT5 and LIF-STAT3 pathways, and then coupling them with a mathematical term that represents the binding of SOCS3 to the PRL receptor. The high-level schema for the model is shown in Figure 1.

RESULTS AND DISCUSSION

In bovine mammary alveolar tissue, pSTAT5 levels were down-regulated 2-fold ($P < 0.01$) by 24 h post-milking and by 192 h this decline was 7.7-fold

FIGURE 1: Schema showing high-level interactions represented in the model. Arrowed truncated lines indicate activation, truncated dashed lines indicate inhibition of activation, solid arrowed lines indicate upregulation.

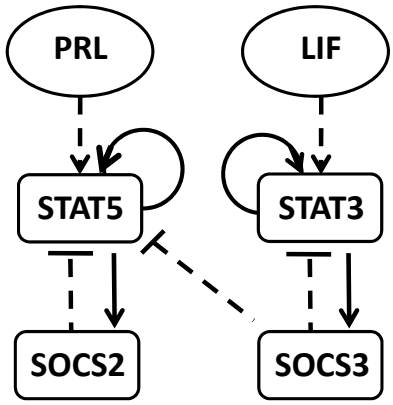
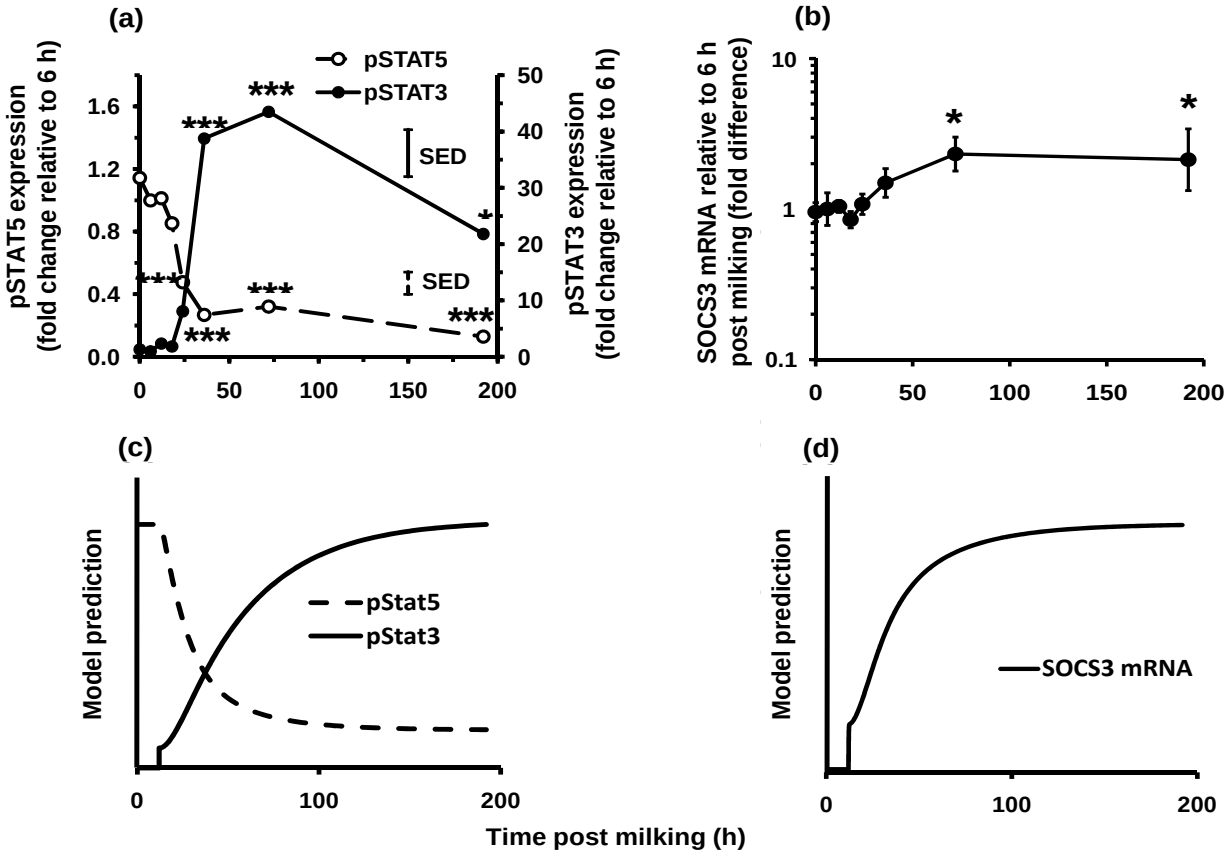


FIGURE 2: Time-course changes during involution in mammary alveolar tissue of lactating cows (n = 6 per time point): (a) Densitometric analysis of pSTAT5 and pSTAT3 protein levels. Data are expressed as mean fold changes with the standard error of difference (SED) relative to the 6 h time point; (b) SOCS3 mRNA levels. Data are expressed as mean fold change ± standard error of mean and significance relative to 6-h time point. Model predictions are graphed qualitatively, without a scale, for (c) pSTAT5 and pSTAT3 levels and (d) SOCS3 mRNA. These predictions suggest that if LIF reduces PRL signalling, then pSTAT3 and SOCS3 expression must remain elevated to maintain suppression of pSTAT5.



(P <0.01), relative to 6 h post-milking. In contrast, pSTAT3 was barely detectable at the early time points and was dramatically up-regulated by 36 and 72 h post-milking by 39- and 44-fold (P <0.001), respectively, and at 192 h post-milking by 22-fold (P <0.05), all relative to 6 h (Figure 2). SOCS3 mRNA was lowly expressed throughout the time-series, although a 2.3-fold increase (P <0.05) was detected by 72 h post-milking, compared to 6 h. Milk protein gene expression begins to decline in bovine mammary by 24 h post-milking (Singh *et al.*, 2008). This is the same time-frame as the pSTAT5 decline, which concurs with a role for STAT5 in lactogenesis (Liu *et al.,* 1997). In rodents, STAT5 also plays a critical role in MEC survival by protecting against STAT3-mediated apoptosis. Furthermore, STAT3 suppresses key survival signals at the onset of involution (Clarkson *et al.*, 2006). Thus, the balance between STAT5 and STAT3 may be important in regulating survival factors in the transition from lactation to involution. In bovine mammary tissue, multiple survival factors are down-regulated 24 h post-milking (Singh *et al.*, 2005). In the present study, SOCS3 was up-regulated at the same time MEC apoptosis was increased at 72 h post milking (Singh *et al.,* 2005). In rodents, a dramatic decline in milk protein expression and reciprocal activation of STAT5 and STAT3 occur by 24 h following the cessation of milk removal. Massive apoptosis begins at 48 h, and at 72 h the gland has entered the irreversible phase of involution (Marti *et al.*, 1999). The present study demonstrates that these changes occur more slowly in bovine mammary tissue, and the small increase in MEC apoptosis at 72 and 192 h post-milking (Singh *et al.*, 2005), suggests the gland is still in the early stages of involution and the irreversible phase has not yet been reached. Milk production can still be fully restored after 7 days of non-milking (Dalley & Davis, 2006). The results from the modelling demonstrated that in the absence of other controlling factors, LIF could indeed reduce STAT5-mediated PRL signalling. Rather than pSTAT5 decreasing progressively as LIF concentration was increased, the model indicated pSTAT5 would be switched from a high to low level once LIF concentration

exceeded a threshold. For the parameterisation used, this threshold occurred when LIF concentration was approximately 10^{-4} that of PRL. However, the model predictions for the gene expression of SOCS3, and the pSTAT3 and pSTAT5 levels did not accord with the measured data. In particular, the model suggested that LIF-upregulated pSTAT3 remains elevated while suppressing pSTAT5, whereas the data showed pSTAT3 fell significantly from 72 h to 192 h, while pSTAT5 declined slightly over the same period of time. This suggests that additional mechanisms are likely to be involved. A candidate has recently been suggested by Tiffen *et al*. (2008), who showed that LIF may act through oncostatin to regulate STAT5 activation. In summary, this study demonstrates that the PRL-STAT5 and LIF-STAT3 pathways play a central role in the early stages of bovine mammary gland involution. However, while STAT3 signalling could potentially attenuate STAT5 signalling, it appears that other factors are also involved. Further investigations may elucidate the underlying mechanisms and lead to the development of intervention strategies to enhance the efficiency of milk production during late lactation in dairy cows.

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