J. Dairy Sci. 99:1–21
http://dx.doi.org/10.3168/jds.2015-10515

Cell survival signaling in the bovine mammary gland during the transition from lactation to involution

K. Singh,*[1,2] I. Vetharaniam,*[1] J. M. Dobson,*[3] M. Prewitz,*[4] K. Oden,*[5] R. Murney,* K. M. Swanson,* R. McDonald,* H. V. Henderson,* and K. Stelwagen†
*AgResearch Ltd., Ruakura Research Centre, Private Bag 3123, Hamilton 3214, New Zealand
†SciLactis Ltd., Waikato Innovation Park, Ruakura Road, Hamilton 3214, New Zealand

ABSTRACT

In dairy cows, mammary gland involution, and thus a decline in milk production, occurs following peak lactation. To examine the cell signaling pathways regulating involution of the mammary gland, signal transducer and activator of transcription factors (STAT5 and 3), suppressors of cytokine signaling (SOCS1–3 and CIS), insulin-like growth factors (IGF1 and 2), and protein kinase B (Akt) were examined. Mammary involution was induced by termination of milking, and alveolar tissue was collected from 52 nonpregnant, primiparous, mid-lactation Holstein-Friesian cows killed at 0, 6, 12, 18, 24, 36, 72, and 192 h postmilking. Qualitative immunohistochemistry showed that activated (phosphorylated) STAT5-P was localized in nuclei of mammary epithelial cells at the early time points, with detection levels decreasing by 24 h postmilking. In contrast, STAT3-P was barely detectable at the early time points, with detection levels increasing following longer postmilking periods. This was supported by Western analysis, which showed a decline in STAT5 and STAT5-P protein levels by 24 h postmilking, no change in STAT3 levels, and an increase in STAT3-P protein (barely detectable at the early time points) by 72 h postmilking. Quantitative real-time reverse transcription PCR analysis showed *SOCS1* and *SOCS3* mRNA increased by 72 h postmilking compared with 6 h postmilking. The *SOCS2* mRNA remained unchanged across the time series, whereas *CIS* decreased by 18 h postmilking and remained lower compared with that at 6 h postmilking until 72 h postmilking. The *IGF1* mRNA increased by 192 h postmilking, whereas *IGF2* mRNA decreased by 18 h postmilking compared with 6 h postmilking. The *IGFBP5* mRNA and protein levels of Akt and Akt-P remained unchanged over the time series. These results show that reciprocal activation of STAT5 and STAT3 occurs at the onset of mammary gland involution in the bovine, albeit at a slower rate than in rodents. Mathematical modeling of the pathways indicated that activated STAT3 could block the STAT5 pathway by upregulating SOCS3. The regulation of IGF1-Akt signaling suggests that by 192 h postmilking in dairy cows, the involution process is still in the reversible phase, with quiescent mammary epithelial cells not yet in the senescent phase.

Key words: dairy cow, mammary gland involution, cell survival, signal transducer and activator of transcription factors

INTRODUCTION

Mammary function and survival of mammary epithelial cells (**MEC**) are not only regulated at the hormonal level (Wilde et al., 1999) but also locally, as responses to the cessation of milk removal demonstrate that intramammary signals play a role in initiating apoptosis of MEC and involution in both rodents (Quarrie et al., 1996; Travers et al., 1996; Li et al., 1997) and ruminants (Quarrie et al., 1994; Wilde et al., 1997). However, involution of the mammary gland is more gradual in the bovine (Holst et al., 1987; Hurley, 1989; Capuco and Akers, 1999) than in rodents. Many alveolar structures are retained (Holst et al., 1987; Hurley, 1989), which may allow for reversibility of involution following extended nonmilking periods in cows (Noble and Hurley, 1999). Furthermore, the increase in apoptosis of MEC following the cessation of milk removal (Wilde et al., 1997; Singh et al., 2005) does not occur to the same extent in the bovine as in rodent mammary glands.

In rodents, mammary gland involution occurs in 2 stages (Lund et al., 1996), with the first stage being

Received October 12, 2015.
Accepted May 1, 2016.
[1]Corresponding authors: kuljeet.singhparhar@outlook.com (for molecular measures and data analysis) and kumar.vetharaniam@agresearch.co.nz (for model development and simulation)
[2]Current address: Science Consultancy, Hamilton 3200, New Zealand.
[3]Current address: Carne Technologies Ltd., Private Bag 740, Cambridge 3450, New Zealand.
[4]Current address: Leibniz Institute of Polymer Research, Dresden, Germany.
[5]Current address: ManukaMed Ltd., 10 Bisley Road, Hamilton 3214, New Zealand.

reversible up to 48 h after pup removal, and the second stage (irreversible from 72 h) being indicated by loss of the majority of MEC by apoptosis (Walker et al., 1989) and extensive restructuring of the gland to a virgin-like state (Jaggi et al., 1996; Lund et al., 1996; Li et al., 1997). The molecular mechanisms regulating MEC apoptosis have been well studied in rodent mammary gland involution. Serine/threonine kinase Akt is a key cell survival factor (Schwertfeger et al., 2001) with multiple intracellular signaling pathways, either up- or downregulated at the onset of mammary gland involution, mediated through the phosphatidylinositol3-kinase-Akt axis (Baxter et al., 2007). Those that are downregulated include prolactin (**PRL**) signaling via the inactivation of signal transducer and activator of transcription (**STAT**)5 (Schmitt-Ney et al., 1992; Liu et al., 1996), insulin-like growth factor (**IGF**) signaling via the upregulation of IGF-binding protein (**IGFBP**)5 (Tonner et al., 1997), and the disruption of cell-extracellular matrix interactions via integrins and the downregulation of focal adhesion kinase (Gilmore et al., 2000; McMahon et al., 2004). In contrast, cell death signaling pathways (Clarkson et al., 2000; Nguyen and Pollard, 2000; Baxter et al., 2006) and immune signaling pathways are activated, including leukemia inhibitory factor-signal transducer (**LIF**), which activates proapoptotic STAT3 (Chapman et al., 1999; Kritikou et al., 2003). The STAT3 activates acute phase response and inflammation related genes (Clarkson et al., 2004) and also IGFBP5, thus providing a link of STAT3 to IGF and Akt survival signaling (Chapman et al., 1999).

The balance between STAT5 and STAT3 signaling plays a role in regulating the transition from lactation to involution. Activation of STAT5 is a survival signal in MEC, suppressing STAT3-mediated apoptosis (Clarkson et al., 2006). Furthermore, LIF negatively modulates STAT5 activation (Granillo et al., 2007). However, the interaction of these STAT signaling pathways at the onset of involution is unclear, although it may be mediated by suppressor of cytokine signaling 3 (**SOCS3**). Activated STAT3 upregulates SOCS3 (Alexander and Hilton, 2004; Clarkson et al., 2006), which then regulates further STAT3 activation to ensure controlled apoptosis of MEC and mammary tissue remodeling during involution (Sutherland et al., 2006; Robinson et al., 2007). In vitro studies show that SOCS3 can inhibit PRL induction of milk protein gene expression and STAT5 activation, through direct interaction with PRL receptors (**PRLR**; Dif et al., 2001). Other members of the SOCS family may be more important in the developing mammary gland, such as cytokine inducible SH-2 domain protein (**CIS**; Tonko-Geymayer et al., 2002), SOCS1 (Lindeman et al., 2001), and SOCS2 (Harris et al., 2006).

The aim of this study was to examine the molecular mechanisms regulating the onset of involution of the bovine mammary gland by investigating the temporal changes in cell survival and death signaling factors and to use mathematical modeling to investigate whether reciprocal patterns of expression between activated STAT5 and STAT3 could arise from cross talk mediated by SOCS3. Such knowledge would provide a greater understanding of the molecular mechanisms that regulate the transition from lactation to involution in the dairy cow. Ultimately, this may inform the development of novel strategies for improving milk production, especially in the later stages of lactation.

MATERIALS AND METHODS

Animals and Tissue Collection

All animal manipulations were conducted in compliance with the rules and guidelines of the Ruakura Animal Ethics Committee (Hamilton, New Zealand). Involution of the mammary gland was induced by abrupt termination of milking in 52 nonpregnant primiparous Holstein-Friesian dairy cows at, or close to, their peak milk production in mid lactation (94 ± 3 DIM). The cows were solely pasture-fed, grazing ryegrass/white clover, and were milked twice daily from parturition. Milking was ceased following 2 consecutive 12-h intervals of milking for the designated nonmilking intervals. Before the cessation of milking, the mean daily milk yield was 14.3 ± 0.3 kg/cow and mean SCC in composite (4 quarters) milk was 165,000 ± 30,000 cells/ml. The experimental design was split across 2 consecutive seasons. In the first season, 42 of the cows (mean 89.1 ± 2.2 DIM), balanced for milk yield, were randomly allocated into 0-, 6-, 12-, 18-, 24-, 36-, and 72-h non-milking-interval groups. In the following season, 10 cows (mean 116.9 ± 6.0 DIM), balanced for milk yield, were randomly allocated into 72- (n = 4) and 192-h (n = 6) non-milking-interval groups. Animals were slaughtered (between 1000 and 1100 h) at 0, 6, 12, 18, 24, 36, 72, and 192 h following the last milking, at the Ruakura Abattoir (Hamilton, New Zealand) using standard commercial procedures (electrical stunning followed by exsanguination). Mammary alveolar tissue (approximately 30 g) was dissected from the middle of the upper one-third of the gland of a rear quarter of each animal, snap-frozen in liquid nitrogen, and stored at −80°C for subsequent RNA and protein extraction (Singh et al., 2005). Approximately 1.5-cm-thick samples of alveolar tissue (10 g) were also obtained from each animal, fixed in 4% phosphate-buffered paraformaldehyde, and processed for histological analysis as previously described (Singh et al., 2005).