BRIEF COMMUNICATION

Induced physical distension of rat mammary glands accelerates the onset of apoptosis and involution compared with milk accumulation alone

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Extended periods of milk accumulation result in reduced milk secretion, increased apoptosis and eventually, involution of mammary glands. The subsequent change in epithelial cell shape from a cuboidal to a flattened morphology as the alveoli distend with milk may initiate changes in protein and gene expression (mechanotransduction) triggering the process of involution (Davis *et al*., 1999). The rapid up-regulation of the pro-apoptotic and immune response marker pSTAT3 (Li *et al*., 1997), the loss of cell-extracellular matrix (ECM) survival signalling via β1-integrin (McMahon *et al*., 2004; Singh *et al*., 2005) and the down-regulation of tight junction (TJ) proteins (occludin, claudin-1 and ZO-1; Cooper *et al*., 2003; 2004) which occur during mammary engorgement are postulated to participate in these mechanotransduction events. Therefore, the aim of this experiment was to investigate the effect of induced physical distension of rat mammary glands on early events during mammary involution.

Sprague-Dawley rats (n=24) at peak lactation each had 2 teats sealed to induce mammary engorgement. Another gland was distended by infusing 0.8 ml of sterile isosmotic (*i.e*. to milk, 300 mOsm; ~6 h worth of milk secretion) sucrose solution up-the-teat prior to sealing. Animals were anaesthetised for the duration of the procedure (10-15 mins) with propofol (15 mg/kg bodyweight i.v. Rapinovet; Schering-Plough Animal Health Ltd., Upper Hutt, NZ). To help visualise the teat orifice and facilitate even distribution of infused sucrose in the gland, milk ejection was induced with 100 mu i.v. oxytocin (Oxytocin V; Vetpharm (NZ) Ltd., Auckland). Nine pups of each litter were returned to their dam and allowed to suckle. The unsealed teats that could be emptied of milk served as controls. Dams were euthanased and mammary tissue collected for control, engorged and infused glands at 0, 1, 3, or 6 h after sealing teats (n= 6 per time point).

Histological analysis of cell death by *in situ* end-labelling (ISEL) of apoptotic nuclei was performed on formalin-fixed mammary sections as described by Singh *et al*. (2005). The number of ISEL nuclei per 100 x magnification field (n=10 per sample) were counted, incremented by 1, and corrected for the number of alveoli per field (*i.e*. 1 + ISEL nuclei per alveolus). Western blotting was performed on mammary protein extracts as described previously (Cooper *et al*., 2004) using polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) against β1-integrin (1:3000) and pSTAT3 (1:2000) for NP-40 detergent soluble protein fractions. Occludin was detected using a polyclonal antibody (Zymed Laboratories Inc., South San Francisco, CA, USA) for both soluble and insoluble fractions (1:80,000 and 1:20,000 dilutions, respectively), as TJ proteins are partially resistant to detergent extraction. Immunoreactive bands were then quantified by densitometry.

All data were $\log_{10}$-transformed and analysed using ANOVA in GenStat (releases 7.1 and 8.1), with blocking on animal to detect differences between treatments at each time point. Data are presented as back-transformed means for control, engorged and infused glands, with the SED between means.

Infusing glands with the equivalent of ~6 hours worth of milk secretion immediately increased alveolar lumen size and flattened/stretched the mammary epithelium, with signs of reduced secretory activity, milk vesicle engorgement, leukocyte invasion and collapsed alveoli apparent within 6 hours (Figure 1A). A low frequency of apoptotic cells was detected in suckled control glands.

In comparison, a dramatic increase ($P<0.001$) in the number of ISEL apoptotic nuclei occurred at 1, 3 and 6 h in infused glands, and at 6 h in engorged glands (Figure 1). By 3 h and at 6 h, apoptosis in infused glands was also greater ($P<0.001$) than in engorged glands. This was accompanied by a marked increase ($P<0.01$) in pSTAT3 expression by 1, 3 and 6 h in infused glands compared with control and engorged glands (Figure 2). In contrast, a reduction (~3-fold) in the expression of β1-integrin in infused glands, compared with control ($P<0.05$) and engorged ($P<0.1$) glands, was detected by 6 h. Occludin expression was

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significantly up-regulated in both soluble and insoluble fractions of infused glands immediately following physical distension at 0 hours compared with control and engorged glands, but then declined to be down-regulated within 6 hours and this was significant compared with control (3-fold) and engorged (2-fold) glands in the soluble protein fraction.

Overall, this study demonstrated that acute physical distension of rat mammary glands accelerated the onset of apoptosis, the up-regulation of the pro-apoptotic marker pSTAT3 and the loss of β1-integrin and occludin protein expression, compared with milk accumulation alone. This procedure would have diluted any potential chemical inhibitory factor(s) in milk (Wilde *et al*., 1995; Shamay *et al*., 2003). Therefore, these results support the hypothesis that physical distension is a primary trigger of mammary apoptosis and involution (Davis *et al*., 1999), and concur with earlier studies in lactating goats and cows which demonstrated that physical distension rather than chemical inhibition was responsible for initiating reductions in TJ integrity and milk secretion during extended periods of milk accumulation (Peaker, 1980; Stelwagen *et al*., 1998).

In the present study the down-regulation of β1-integrin and occludin expression following induced physical distension indicate a loss of cell-ECM communication through focal adhesion complexes and cell-cell integrity through TJ complexes, respectively, consistent with that reported during the initial, apoptotic, phase of mammary involution (Cooper *et al*., 2003; 2004; McMahon *et al*., 2004; Singh *et al*., 2005). However, occludin expression was initially dramatically up-regulated following the acute physical distension and this may reflect an attempt to maintain mammary TJ integrity before the system was overwhelmed. Rapid increases in occludin expression, presumably to enhance TJ synthesis and repair, have also been reported in response to TJ breakdown by low-calcium conditions in mouse mammary epithelial cells *in vitro* (Stelwagen & Callaghan, 2003).

The changes in occludin and β1-integrin expression following physical distension of the mammary epithelium support the theory that cell-cell and cell-ECM contacts act as mechanosensors and/or participate in mechanotransduction pathways, as demonstrated in other cell types (Cavanaugh *et al*., 2001; Shyy & Chien, 1997). Furthermore, the rapid increase in pSTAT3, a regulator of mammary apoptosis and the acute phase immune response (Li *et al*., 1997), indicate that it also participates in mechanotransduction events. Therefore, this study supports further investigation of a role for mechanotransduction during apoptosis and involution of mammary glands.

Figure 1: *In situ* end-labelling (ISEL) of apoptotic nuclei in control, engorged, and infused mammary glands at 0, 1, 3, and 6 h (n=6 rats per time point) following teat-sealing. (A) Representative histological sections at 6 h (200 x magnification; scale bar equals 100 μm). Examples of positive ISEL apoptotic nuclei located in epithelia (→) or lumina (single or regions●→) are shown and sections are counterstained with nuclear fast red. Apoptotic nuclei were not labelled in negative controls. (B) Back-transformed mean number of 1 + ISEL nuclei per alveolus with the SED to compare treatments at each time point (*P<0.05, ***P<0.001 relative to controls, } = P-value significant for infused glands relative to engorged glands).

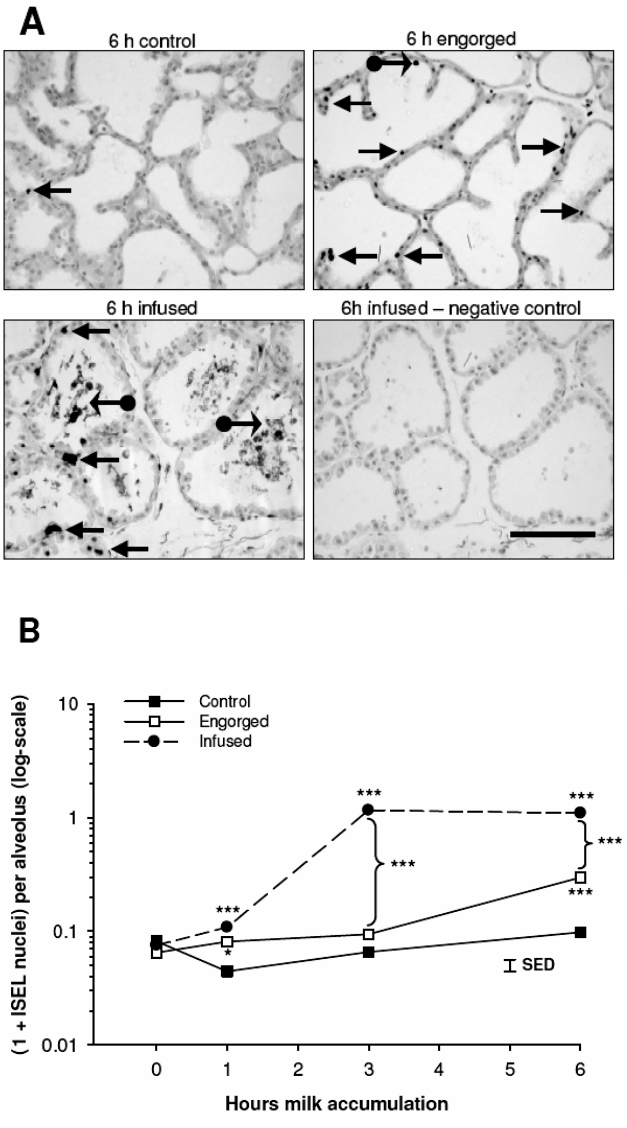
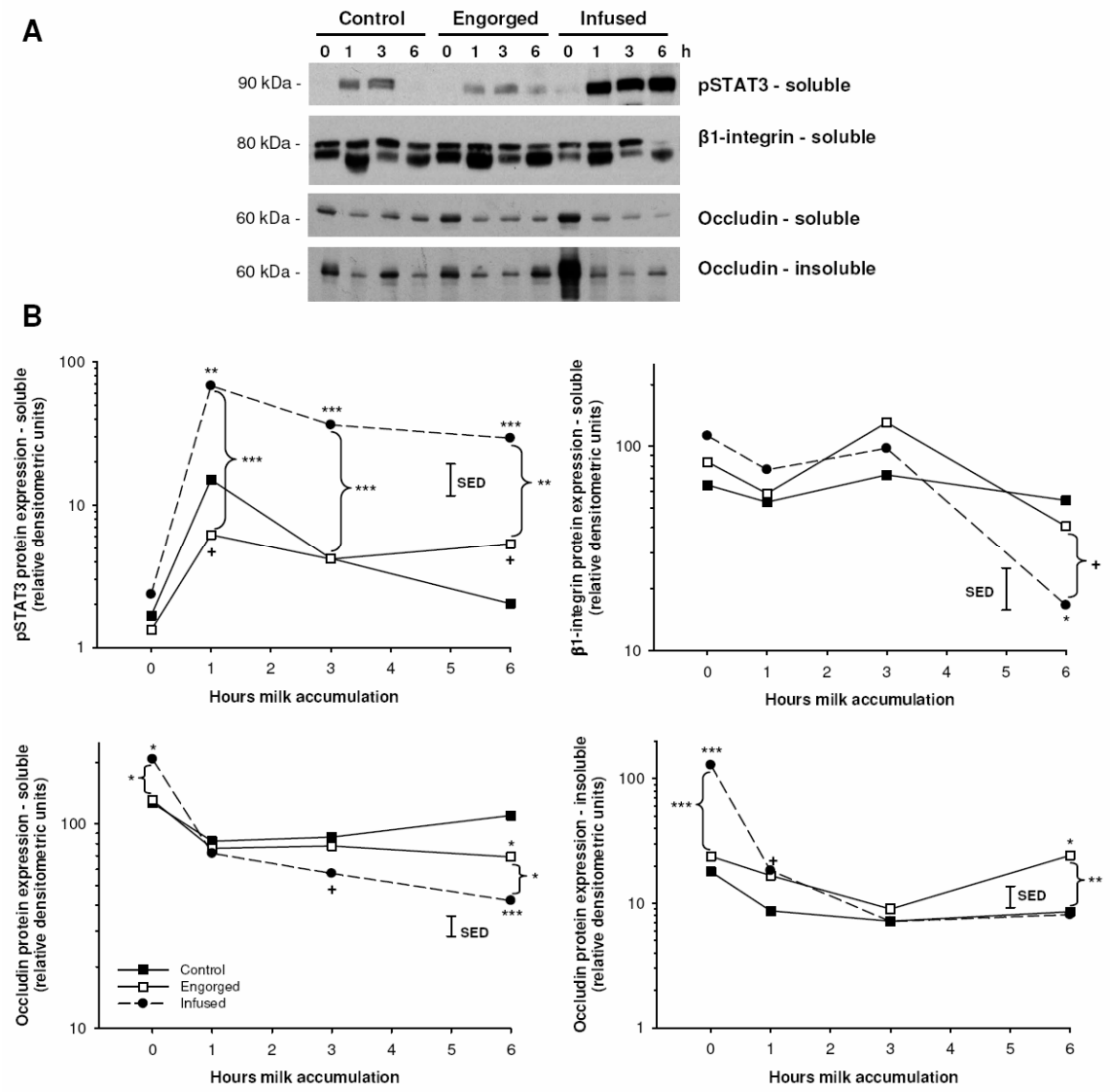


Figure 2: Representative western blots (A) and densitometric analysis (B) of pSTAT3 and β1-integrin expression in soluble protein fractions and occludin in soluble and insoluble protein fractions from control (■, solid line), engorged (□, solid line) and infused (●, dashed line) mammary glands at 0, 1, 3, and 6 h (n=6 rats per time point) following teat-sealing. Results are presented as back-transformed means with the SED to compare treatments at each time point ($^{+}$ P<0.1, *P<0.05, **P<0.01, ***P<0.001 relative to controls, } = P-value significant for infused glands relative to engorged glands). Note: β1-integrin analysis refers to the top immunoreactive band at ~80 kDa, which aligns with positive controls (results not shown).



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REFERENCES

Cavanaugh, K.J. Jr.; Oswari, J.; Margulies, S.S. 2001: Role of stretch on tight junction structure in alveolar epithelial cells. *American Journal of Respiratory Cell and Molecular Biology* **25**: 584-591.

Cooper, C.V.; Stelwagen, K.; McMahon, C.D.; Singh, K.; Farr, V.C.; Davis, S.R. 2003: Tight junction (TJ) protein expression during engorgement of rat and bovine mammary glands. *Journal of Dairy Science* **86** (Supplement 1): 33.

Cooper, C.V.; Stelwagen, K.; Singh, K.; Farr, V.C.; Prosser, C.G.; Davis, S.R. 2004: Expression of the tight junction protein zonula occludens-1 during mammary engorgement. *Proceedings of the New Zealand Society of Animal Production* **64**: 43-47.

Davis, S.R.; Stelwagen, K.; Farr, V.C. 1999. Regulation of yield loss and milk composition during once-daily

milking: a review. *Livestock Production Science* **59**: 77-94

Li, M.; Liu, X.; Robinson, G.; Bar, P.U.; Wagner, K.U.; Young, W.S.; Hennighausen, L.; Furth, P.A. 1997: Mammary-derived signals activate programmed cell death during the first stage of mammary gland involution. *Proceedings of the National Academy of Sciences of the United States of America* **94**: 3425-3430.

McMahon, C.D.; Farr, V.C.; Singh, K.; Wheeler, T.T.; Davis, S.R. 2004: Decreased expression of ß1-integrin and focal adhesion kinase in epithelial cells may initiate involution of mammary glands. *Journal of Cellular Physiology* **200**: 318-325.

Peaker, M. 1980: The effect of raised intramammary pressure on mammary function in the goat in relation to the cessation of lactation. *Journal of Physiology* **301**: 415-428.

Shamay, A.; Shapiro, F.; Leitner, G.; Silanikove, N. 2003: Infusions of casein hydrolyzates into the mammary gland disrupt tight junction integrity and induce involution in cows. *Journal of Dairy Science* **86**: 1250-1258.

Shyy, J.Y.; Chien, S. 1997: Role of integrins in cellular responses to mechanical stress and adhesion. *Current Opinion in Cell Biology* **9**: 707-713.

Singh, K.; Dobson, J.; Phyn, C.V.C.; Davis, S.R.; Farr, V.C.; Molenaar, A.J.; Stelwagen, K. 2005: Milk accumulation decreases expression of genes involved in cell- extracellular matrix communication and is associated with induction of apoptosis in the bovine mammary gland. *Livestock Production Science* **98**: 67-78.

Stelwagen, K.; Callaghan, M.R. 2003: Regulation of mammary tight junctions through parathyroid hormone-related peptide-induced activation of apical calcium channels. *Journal of Endocrinology* **178**: 257-264.

Stelwagen, K.; Farr, V.C.; Davis, S.R.; MacFadden, H.A. 1998: Inhibition of milk secretion and the extent of filling of the bovine mammary gland. *Journal of Dairy Science* **81** (Supplement 1): 376.

Wilde, C.J.; Addey, C.V.; Boddy, L.M.; Peaker, M. 1995: Autocrine regulation of milk secretion by a protein in milk. *The Biochemical Journal* **305**: 51-58.