Expression of the tight junction protein zonula occludens-1 during mammary engorgement

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ABSTRACT

An increase in tight junction (TJ) permeability occurs during extended milking intervals and is associated with reduced milk secretion. This paper examines temporal changes in the expression of a major TJ protein, zonula occludens-1 (ZO-1), during mammary engorgement. Alveolar mammary tissue was collected from 52 mid-lactation dairy heifers at 0, 6, 12, 18, 24, 36 and 72 h, and at 8 d following abrupt cessation of milking (n = ≥ 6 cows per time point). ZO-1 protein expression significantly decreased during mammary engorgement ($P<0.05$). Similarly, ZO-1 mRNA expression had halved by 36 h to 8 d post-milking ($P<0.05$). In a second trial, alveolar tissue was collected from the udder halves of late-lactation dairy cows (n = 4) that were milked unilaterally, either once-daily or twice-daily for 4 days. A 1.7-fold increase in ZO-1 mRNA expression was detected after once-daily milking, compared with twice-daily milking ($P<0.05$). The down-regulation of ZO-1 during mammary engorgement is consistent with increased TJ permeability. However, the up-regulation of ZO-1 mRNA expression during short-term once-daily milking suggests that TJ function is repaired as the mammary gland adjusts to the extended milking interval. In conclusion, we have identified changes in ZO-1 critical to TJ function in bovine mammary glands.

Keywords: bovine; mammary; once-daily milking; tight junction; ZO-1.

INTRODUCTION

Tight junctions (TJs) are the most apical member of the junctional complex between adjacent cells in various epithelia, including the mammary gland. They surround each cell, forming gasket-like seals to prevent the paracellular movement of ions and small molecules between the interstitial fluid and milk ('barrier function'). In addition, the TJ contributes to polarised milk synthesis and secretion by separating the plasma membrane into apical and basolateral domains of distinct protein and lipid composition ('fence function').

An increased TJ permeability occurs during reduced milking frequencies, e.g., once-daily milking (ODM) (Stelwagen *et al*., 1994), and involution (Fleet & Peaker, 1978). Milk yield is decreased by 7-34% during ODM, which is commonly practised by New Zealand dairy farmers as a management tool during late lactation (Davis *et al*., 1999). While the decline in milk secretion rate during extended periods of milk accumulation coincides with the rise in TJ permeability (after approximately 18 h) (Stelwagen *et al*., 1997), the exact mechanism through which leaky TJs affect milk production remains unclear. However, a direct link is suggested by experiments showing that milk yield can be reduced by a similar amount to that observed during ODM by disrupting TJs using the calcium chelator, EGTA (Stelwagen *et al*., 1995).

Stretching of the mammary epithelium during milk accumulation has been postulated to initiate gene expression events (mechanotransduction) that culminate in disruption of TJ function (Stelwagen *et al*., 1997). This mechanism is likely to affect the function and composition of molecules present in the TJ complex. Occludin and claudins are integral transmembrane TJ proteins that bind to the cytoplasmic proteins zonula occludens (ZO)-1, ZO-2 and ZO-3, providing the TJ with structural and signalling links to the cell interior (reviewed by Fanning *et al*., 1999). Importantly, ZO-1 links occludin to the actin cytoskeleton (Fanning *et al*., 1998) and is involved in signal transduction at the TJ (Meyer *et al*., 2003). We have previously reported the down-regulation of occludin and claudin-1 protein expression during milk accumulation in rat and bovine mammary glands (Cooper *et al*., 2003), and hypothesise a similar down-regulation of ZO-1 in a mechanotransduction cascade that initiates increased TJ permeability. The objective of the present study was, therefore, to determine the effect of mammary engorgement on the expression of ZO-1 in bovine mammary glands.

MATERIALS AND METHODS

Animals and tissue collection protocols

Experiment 1 – Time-course following abrupt cessation of milking.

The first lactation of 52, non-pregnant Friesian dairy heifers (94.0 ± 3.0 mean days in milk; average daily milk yield, 14.3 ± 0.3 kg/cow) was abruptly ceased by stopping milk removal. Animals were then slaughtered at 0, 6, 12, 18, 24, 36 and 72 h, and at 8 d (n = ≥ 6 cows per time point) following the last morning milking.

Experiment 2 - Short-term once-daily milking.

Four non-pregnant multiparous Jersey and Jersey x Friesian crossbred dairy cows in late lactation (185.5 ± 20.6 mean days in milk) were used in this study. The left and right udder halves of each cow were randomly assigned to two milking frequencies; either twice-daily

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milking (TDM) or ODM for 4 days. Animals were not milked on the fourth morning and were killed for tissue collection. Milk yields were measured for each udder half 1 day before and 3 days after the start of unilateral milking.

All procedures used in experiments 1 and 2 above were approved by the Ruakura Animal Ethics Committee. Animals were slaughtered at the Ruakura Abattoir using standard commercial methods. Alveolar mammary tissue was immediately collected post-mortem and frozen on liquid nitrogen before storage at -80 °C.

RNA extraction and cDNA synthesis

Total RNA was extracted from ground tissue using TRIzol (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. RNA was treated with DNaseI (Invitrogen) and column-purified using a QIAGEN RNeasy Mini Kit (QIAGEN Sciences, Germantown, MD, USA) according to the manufacturers' instructions. cDNA was generated from the purified RNA using the Superscript II Reverse Transcriptase First-Strand Synthesis System protocol (Invitrogen).

Real-time RT-PCR

Quantitative real-time RT-PCR using the relative standard curve method, and with SYBR Green Master Mix, was carried out in the ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA) as directed by the manufacturer. Briefly, cDNA samples were PCR amplified for ZO-1 (primers: forward 5'-GAT TCA CAC CAA AAC CAT ACA C-3' and reverse 5'-CGT CTT CGT CTT CAT CTT CCT-3') and ubiquitin (primers: forward 5'-GGC AAG ACC ATC ACC CTG GAA- 3' and reverse 5'-GCC ACC CCT CAG ACG AAG GA- 3') products under the following conditions: 95 °C for 10 min (initial denaturing), then 40 cycles of 95 °C for 15 s (denaturing), 56 °C for 30 s (annealing), 72 °C for 30 s (extending) and 78 °C for 10 s (measuring). Dissociation curve analysis and gel electrophoresis of amplified products confirmed the specificity of each real-time reaction. The authenticity of amplified products was also confirmed by sequencing (Waikato DNA Sequencing Facility, Hamilton, NZ).

Protein extraction

Ground tissue was homogenised in low-salt buffer, containing 1% Nonidet P-40 (NP-40) detergent, essentially as described previously (McMahon *et al*., 2004). The homogenate was centrifuged at 10,000 *g* for 30 min and the supernatant collected as the NP-40-soluble protein fraction. To collect the NP-40-insoluble protein fraction, the remaining pellet was resuspended by sonication in low-salt buffer containing 1% NP-40 detergent and 1% sodium dodecyl sulphate (SDS). Samples were mixed with loading buffer, boiled for 5 min, and stored at -20 ˚C until further use (Laemmli, 1970).

Western blotting

Proteins were separated by 7% SDS-polyacrylamide gel electrophoresis (PAGE) (Laemmli, 1970) and transferred to a nitrocellulose membrane. Membranes were subjected to immunoblotting as described previously (McMahon *et al*., 2004), except that the primary antibody used was rabbit anti-human ZO-1 (Zymed Laboratories Inc., South San Francisco, CA, USA). Films were scanned and immunoreactive bands subjected to densitometric analyses (GS-800, Bio-Rad Laboratories Pty. Ltd., Auckland, NZ)

Data Analysis

Data were analysed by ANOVA using GenStat 6.0 (Lawes Agricultural Trust, 2002). Relative quantification of gene expression following real-time PCR was performed using the standard curve method (PE Applied Biosystems). The amount of ZO-1 in each sample was normalised to the amount of ubiquitin to control for the initial concentration of cDNA, and the resulting values log-transformed. Densitometry results from western blotting were log-transformed and adjusted for between gel variations. Differences between means at each time point in experiment 1 were evaluated using the Contrasts option of ANOVA. Treatment effects in experiment 2 were analysed within cow, and milk yields during unilateral milking were adjusted for differences in pre-treatment yields between udder halves. Results are expressed as back-transformed mean fold changes relative to the 6 h mean (experiment 1) or the TDM mean (experiment 2). In experiment 1, 6 h post-milking was designated as the calibrator sample as it was the most representative of actively lactating mammary glands. Data are reported as means ± the standard error of the mean (SEM).

RESULTS

Experiment 1

ZO-1 mRNA expression following forced involution

Quantitative real-time RT-PCR was used to determine temporal changes in mammary ZO-1 mRNA expression up to 8 d after the abrupt cessation of milking (Fig. 1). Expression of ZO-1 mRNA was highest at 6 h and at 18 h post-milking. However, ZO-1 mRNA expression had significantly decreased ($P<0.05$) by 36 h following the last milking compared with 6 h (1.9-fold decrease). There was no further decrease from 36 h to 8 d involution ($P>0.05$).

ZO-1 protein expression during mammary engorgement

Western blotting was used to determine the pattern of ZO-1 protein expression in NP-40-soluble (Fig. 2a) and NP-40-insoluble (Fig. 2b) fractions during the early stages of mammary engorgement (up to 72 h post-milking). Immunoreactive bands to ZO-1 were detected at ~225 kDa in the bovine mammary samples. There was a marked decline in ZO-1 protein expression in the NP-40-soluble fraction after 36 to 72 h of mammary engorgement (2.7-fold decrease; $P<0.05$). A multiple

banding pattern and an upwards shift in the size of the 225 kDa bands, consistent with phosphorylation, was evident in the NP-40-insoluble fraction. The decline in ZO-1 expression in the NP-40-insoluble protein fraction was variable during mammary engorgement, but a 2.6-fold decrease was still apparent by 72 h post-milking.

FIGURE 1: Pattern of mammary ZO-1 mRNA expression up to 8 d after the forced involution of Friesian dairy heifers in mid-lactation. Results are expressed as mean fold differences (± SEM) relative to 6 h lactating mammary tissue (n = ≥ 6 cows per time point).

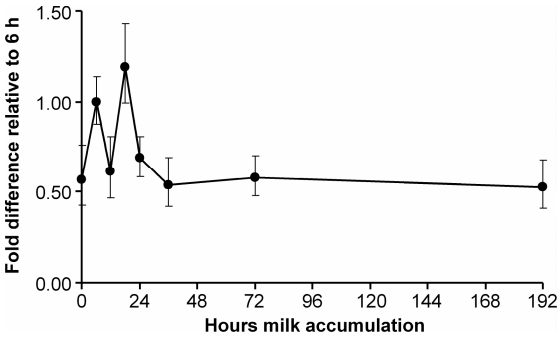
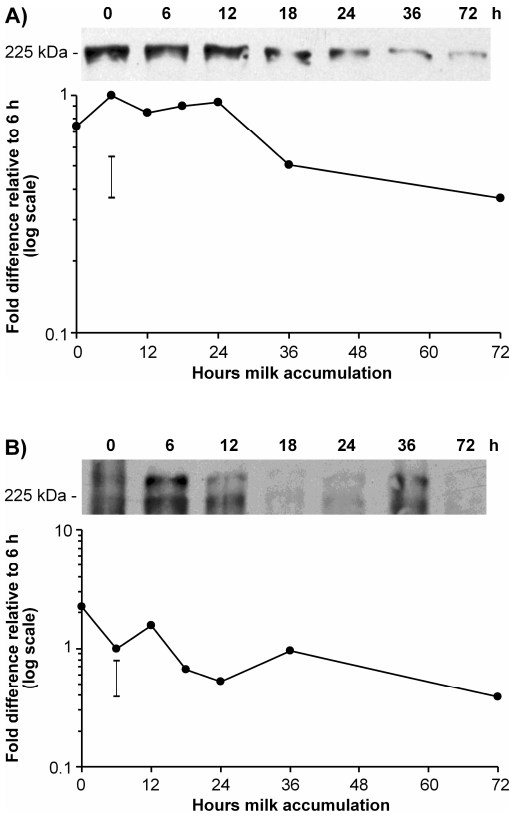


FIGURE 2: Densitrometric analyses of western blots of ZO-1 protein expression in NP-40-soluble (**A**) and NP-40-insoluble (**B**) fractions during the engorgement of bovine mammary glands. Results are graphed as mean fold differences relative to 6 h lactating mammary tissue (n = 6 cows per time point) with the standard error of the difference (SED). A representative western blot is included for both fractions.

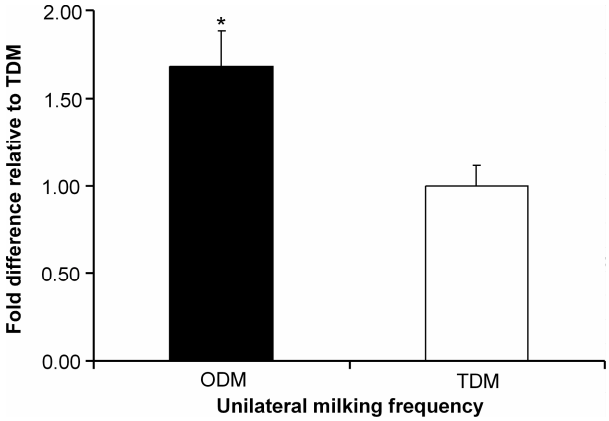


Experiment 2

ZO-1 mRNA expression during once-daily milking

Mixed-age dairy cows in late lactation (average total daily milk yield, 12.7 ± 1.4 kg milk/cow) were milked unilaterally either once- or twice-daily for 4 days. Milk yields, adjusted for pre-treatment values, were decreased by 21% in ODM glands compared with TDM glands ($P<0.05$). There was a 1.7-fold increase in ZO-1 mRNA expression in ODM udder halves compared with TDM controls ($P<0.05$) (Fig. 3).

FIGURE 3: Fold changes (mean ± SEM) in ZO-1 mRNA expression in once-daily milked (ODM) udder halves compared with twice-daily milked (TDM) udder halves from Jersey and Jersey x Friesian crossbred dairy cows (n = 4). Significant differences between ODM and TDM glands are indicated by *$P<0.05$.



DISCUSSION

An increase in mammary TJ permeability occurs during extended periods of milk accumulation and is associated with reduced milk secretion (Stelwagen *et al*., 1995; 1997). The current investigation shows that the disruption of TJ integrity is accompanied by a decrease in ZO-1 mRNA and protein expression, in agreement with our previous results reporting a down-regulation of occludin and claudin-1 in rat and bovine mammary glands (Cooper *et al*., 2003). The decline in ZO-1 expression was significant by 36-72 h following the last milking, which occurs after the reported rise in TJ permeability at 18 h post-milking (Stelwagen *et al*., 1997). Of interest, therefore, was a temporary peak in ZO-1 mRNA expression after 18 h post-milking, which may reflect an attempt to maintain mammary TJ integrity. Increased occludin protein expression, presumably to enhance TJ synthesis and repair, was also reported in response to TJ breakdown by low-calcium conditions in mouse mammary cell lines *in vitro* (Stelwagen & Callaghan, 2003).

Mammary ZO-1 expression was approximately halved after 36 h post-milking and these levels were maintained for up to 8 d involution. This result indicates that the mammary gland retains some residual TJ structure that can be called upon during re-initiation of lactation. This is supported by observations that lactation

can still be reinstated after 11 d of involution (Noble & Hurley, 1999) and that the rapid widespread remodelling and apoptosis occurring during rodent mammary involution (Marti *et al*., 1997) is not evident in the bovine mammary gland (Holst *et al*., 1987).

To further examine temporal patterns of ZO-1 expression during milk accumulation, we separated mammary protein samples into NP-40 detergent-soluble and -insoluble fractions. The resistance of TJ proteins to solubility in detergent-salt extractions indicates their incorporation into the TJ-cytoskeleton complex and is associated with reduced TJ permeability (Sakakibara *et al*., 1997). We report that in the bovine mammary gland, ZO-1 was detected in both detergent-soluble and -insoluble fractions, and that the higher molecular mass of the insoluble protein fraction is consistent with the phosphorylated form of ZO-1 enriched in TJs (Chen *et al*., 2000). A decline (~2.5-fold) in ZO-1 protein expression was detected in both fractions during mammary engorgement. Furthermore, reduced levels of ZO-1 protein expression are associated with TJ disruption in other cell types (Tian & Phillips, 2002).

Therefore, we suggest that the down-regulation of ZO-1, along with occludin and claudin-1 (Cooper *et al*., 2003), after ~36 h of milk accumulation contributes to the loss of TJ integrity, which we postulate to be initiated by alveolar stretch-induced mechanotransduction. However, the response appears to be dependant on the duration and magnitude of engorgement as in contrast to extended milk accumulation, short-term ODM, where alveolar distension is relieved by milk removal every 24 h, caused a 1.7-fold increase in ZO-1 mRNA expression. This suggests evidence of TJ repair as the mammary gland adjusts to the reduced milking frequency. Indeed, the dramatic increases in plasma lactose and α-lactalbumin concentrations that indicate loss of TJ integrity during the initial 24 h of ODM are reduced significantly with each successive milking, implying that the barrier function may be partially restored during ODM (Stelwagen *et al*., 1997).

The mechanisms by which these changes occurred have yet to be determined. However, evidence of a local mechanism is supported by experiment 2, where increased ZO-1 mRNA expression was accompanied by a 21% reduction in milk yield in the ODM glands only. This milk production loss is typical of those reported previously in late lactation (Davis *et al*., 1999). A local mechanotransduction pathway may involve signalling to and from the TJ complex by ZO-1, as this protein is known to participate in signal transduction in other epithelia (Meyer *et al*., 2002). Furthermore, this mechanism may be related to loss of the integrin survival signal from the extracellular matrix during rat (McMahon *et al*., 2004) and bovine (Singh *et al*., this volume) mammary involution, through a common link via the actin cytoskeleton.

In conclusion, this study suggests milk accumulation in bovine mammary glands results in loss of TJ integrity through a pathway involving changes in the synthesis of the structural protein, ZO-1. Further work is required to determine the mechanisms by which occludin, claudins and ZO-1 are regulated during mammary engorgement.

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